Role of Nitric Oxide in Domoic Acid Induced Hippocampal Degeneration

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Abstract

We used a mouse model of domoic acid (DA)-induced temporal lobe epilepsy to determine if nitric oxide (NO) has anticonvulsant properties. The protective effects of NO were assessed using acute and chronic doses of DA (ip) in the presence and absence of NO synthase (NOS) inhibitors. The Institute of Cancer Research mice treated with different doses of DA (1-6mg/kg body weight) showed dose dependent stereotypic neurological signs, which were enhanced by pretreatment with NOS inhibitors. Further, mice sacrificed after 30 and 60 days of DA treatment showed reactive glial cells with large cell bodies and long processes in CA1 and CA2 regions of the hippocampus. These changes were more pronounced in groups treated with neuronal NOS inhibitor especially in 60 days treatment group. In addition, immunohistochemistry and Western blot analysis demonstrated loss of calcium binding protein calbindin D28k immunoreactivity in the hippocampal regions of animals treated with DA plus NOS inhibitors reflecting damage to calbindin rich neurons in the hippocampus. Our findings suggest that a single sub-acute dose of DA can cause progressive damage to the hippocampus, which is markedly influenced by NOS inhibitors. Further, this study establishes that NO agonists may have a therapeutic value in treating patients with temporal lobe epilepsy.

Keywords: Epilepsy, neurodegeneration, hippocampus, domoic acid, excitotoxicity, nitric oxide

Özet

Nitrik oksitin (NO) antikonvülzant özellikleri olup olmadığını araştırmak üzere domoik asit (DA) ile tetiklenmiş fare temporal lob epilepsi modelini kullandı. NO-sentetaz (NOS) inhibitörlerinin varlığı ve yokluğunda DA’ın akut ve kronik dozları kullanılarak NO’nun koruyucu etkileri değerlendirildi. NO’nun koruyucu etkisi akut ve kronik DA dozlarında ve NO sentetaz (NOS) inhibitörlerinin varlığı ve yokluğunda araştırıldı. Değişik DA dozları (1-6mg/kg) ile tedavi edilen ICR farelerinde NOS inhibitörleri ile ön tedavi sonrası kuvvetlenen, doza bağlı stereotipik nörolojik bulgular gözlandı. Farelerde DA ile tedavisinin 30. ve 60. günlerinde yapılan inceleme lerde büyük hücre gövdeleri olan ve hipokampüsün CA1 ve CA2 bölgelerine uzun oluşumları olan reaktif gliyal hücreler saptandı. Bu özellikler nöral NOS inhibitörleri ile tedavi edilen ve özellikle 60 günlük tedavi alan grupta daha belirgindid. İmmunohistokimyasal ve Western blot analizleri ile DA ile NOS inhibitörleri ile tedavi gören deney hayvanlarının hipokampüslerinde kalsiyum bağlı protein D28k immunoreaktivitesine karşı saptandı ki bu durum hipokampüsteki calbindinden zengin nöronlarda hasar bulgusu olarak değerlendirildi. Bulgularımız tek sub-akut DA dozunun hipokampusta NOS inhibitörleri ile birlikte artan ilerleyici hasara neden olduğunu göstermektedir. Ayrıca bu çalışma temporal epilepsili hastaların tedavisinde NO agonistlerin rolü olabileceğini düşündürmektedir.

Anahtar Kelimeler: Epilepsi, nörodejenarasyon, hipokampüs, domoik asit, nitrik oksit
INTRODUCTION

Temporal lobe epilepsy is a common disorder of the brain. This is often associated with characteristic neuronal loss and gliosis in the CA1 and CA3 regions of the hippocampus (12,3). The changes in the hippocampus are referred to as mesial temporal sclerosis (MTS). MTS is the most common lesion to be found in the post mortem brains of patients with TLE who die a natural death. Pathogenesis of MTS remains unknown, however, early life insults such as prolonged febrile convulsions in infancy are thought to be responsible for neuronal damage, which subsequently is presumed to result in a potent epileptogenic lesion (12,3,23,22). Tissues from patients undergoing epileptogenesis are not easily available for studies, which is why it is critical for the development of clinically relevant animal models of TLE. The animal models can help fill the gaps in the understanding of the mechanism of epileptogenesis, as well as in the development and testing of new drugs for effective treatment.

Domoic acid (DA) isolated from contaminated mussels and diatom bloom is a potent neurotoxin and produces hippocampal excitotoxicity similar to kainic acid (29,7). DA also produces structural and functional lesions in different regions of hippocampus in animals treated with DA (9,27). Furthermore, humans accidentally exposed to DA showed lesions similar to MTS (32,6,16). Therefore, the animal model of DA-induced epilepsy appears to be an important tool for understanding the pathogenesis of TLE in humans.

Nitric oxide (NO) has been considered as a neuronal messenger in the central nervous system and modulator of several brain functions (5,8,34,13). NO is synthesized by three distinct mammalian NO synthase isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS), which are constitutively expressed in neuronal and endothelial cells, respectively, and are also collectively referred to as constitutive NOS (cNOS); inducible NOS (iNOS), which is expressed in inflammatory cells (e.g. macrophages and microglia) in response to stimulation by cytokines and/or endotoxins (14,21). In the brain, NO regulates cerebral blood flow, vascular tone and acts as a signaling molecule for synaptic potentiation and plasticity; it also mediates glutamate toxicity (2). Among others, its role in convulsive phenomena has been widely studied in different experimental models, however, the reported results are contradictory. Since the debate as to whether NO has a proconvulsant or anticonvulsant role in seizure activity is still on, the present study was initiated to investigate the role of NO in mouse model of TLE.

METHODS

Materials: Domoic acid and antibodies against calbindin-D28k (CaB) (monoclonal, clone CL-300) were obtained from Sigma (St. Louis, MO, USA). Antibodies against β-III tubulin (monoclonal) were purchased from Promega (Madison, WI, USA). Protein Assay kits were purchased from Bio-Rad (Melville, NY, USA). Western Breeze kits for immunoblotting were obtained from Western Breeze, Invitrogen, Carlsbad, CA, USA, Apoptag (Oncor) in situ apoptosis kit was purchased from FD Neurotechnology inc. (Ellicott city, MD).

Animals and Treatment:

Animals

Outbred, Institute of Cancer Research (ICR) mice (males 8-10wks old, weighing 25-30mg) were obtained from Harlan Sprague Dawley Laboratories. The animals were maintained on a commercial diet and water ad libitum. The use of animals was approved by the Institutional Animal Care and Use Committee.

Treatments

Three sets of experiments were performed in order to study the acute and long term
effects of DA in the presence and absence of NO inhibitors.

**Acute Study**

**Experiment 1** – Dose response of DA treatment. Animals were divided into following groups. A minimum of 3-4 animals were used per group.

- Control Group: Received normal saline (ip)
- DA-treated Group: Received 1, 1.5, 3, 4, and 6mg/kg DA (ip) respectively

**Experiment 2** – Different DA doses (selected from experiment 1) plus NOS inhibitors [N-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI)] were used in experiment 2. ICR mice were divided into following groups. A minimum of 3-4 animals were used per group.

- Group I: Received L-Arginine (1000mg/kg, ip)
- Group II: Received L-Arginine (1000mg/kg, ip) + DA (1mg/kg, ip)
- Group III: Received L-NAME (10mg/kg, ip)
- Group IV: Received L-NAME (10mg/kg, ip) + DA (2mg/kg, ip)
- Group V: Received 7NI (50mg/kg, ip)
- Group VI: Received 7NI (50mg/kg, ip) + DA (2mg/kg, ip)
- Group VII: Received 7NI (50mg/kg, ip) + DA (4mg/kg, ip)

L-NAME and 7NI were given 30 min and L-Arginine was given 5 min prior to DA administration. Animals in all groups were observed for 35-40 min for the stereotypic neurological signs in the form of scratching and for clonic seizures. Groups were terminated after 24 hrs of treatment. Some animals were sacrificed after 6hrs for TUNNEL assay.

**Chronic Study**

The animals were divided into different groups (n=12) and a single sub-acute dose of DA (2mg/kg, ip) along with NOS inhibitors were administered to mice according to following treatment protocol.

- Group I: (Control) received normal saline
- Group II: DA (2mg/kg;ip)
- Group III: L-NAME (10mg/kg, ip) + DA (2mg/kg, ip)
- Group IV: 7NI (50mg/kg, ip) + DA (2mg/kg, ip)

Half of the animals were sacrificed after 30 days and rest after 60 days of treatment. The brains were removed, divided sagittally into halves and one half was frozen at –80°C for biochemical analysis whereas the other half was fixed in phosphate buffered 10% formalin.

**Preparation of NOS Fraction**

To determine the effect of various concentrations of DA on nNOS activity in vitro, the frozen brain samples were homogenized in five volumes of ice-cold homogenizing buffer [Hepes 20mM pH 7.2; containing sucrose 0.32M, EDTA 0.5mM and dithiotheritol 1mM]. After centrifugation of the homogenates at 20,000xg for 15 minutes the cystolic fraction was passed through a 0.75 ml column of Dowex AG 50WX-8 (Na+form) to remove endogenous arginine. The resultant supernatant was used for subsequent assays as an enzyme source.

Protein concentrations were determined according to the method of Bradford (4) using bovine serum albumin (BSA) as standard.

**NOS Activity**

NOS activity in brain samples was determined by measuring the conversion of \[^{3}H\] L arginine to \[^{3}H\] L-citrulline (5). Briefly, each preparation (400 µg protein) was incubated with L-[2,3-\(^3\)H] arginine (10mM, 5GBq/mmol) in the presence of NADPH (0.5mM) and Ca\(^{2+}\) (1µM free calcium) in a total of 400 µl. After 30min incubation at 37°C, the reaction was determined by addition of 0.2 ml of HEPES buffer (100mM, pH 5.5) containing EGTA (10mM). The incubations were then applied to 1ml Dowex AG 50WX-8 columns (Na+ form, Bio-Rad) and the eluted [3H] L-citrulline by liquid scintillation counting.

**TUNEL Assay**
To investigate if acute dose of DA (5mg/kg, ip) causes damage to hippocampal neurons we used Apoptag (oncor) in situ apoptosis detection kit to identify the apoptotic cells in 10µm frozen hippocampal sections of the control and DA treated animals. Briefly, residues of digoxigenin-nucleotide are catalytically added to the DNA of the apoptotic cells by terminal deoxynucleotidyl transferase (TdT). The incorporated nucleotides form a random heteropolymer of digoxigenin -II dUTP and dATP, in a ratio that has been optimized for anti digoxigenin antibody binding. The anti digoxigenin antibody fragment carries a conjugated peroxidase to the reaction site. The localized peroxidase enzyme then catalytically generates an intense signal from chromogenic substrate. The sections were counter stained with 0.5% methyl green.

**Immunohistochemistry**

The formalin fixed sections of hippocampus were immunohistochemically stained with an antibody to glial fibrillary acidic protein (GFAP), beta-III-tubulin and calbindin (CaB) as reported earlier (4,35). Briefly, 6 μm thick tissue sections were deparaffinized, microwaved for 14 min and then incubated with 5% blocking goat serum for 10 min with thorough intervening washes with phosphate-buffered saline (PBS). After washing the sections with PBS, the sections were incubated overnight at 4°C with anti GFAP [monoclonal, Sigma; diluted 1:1000 in 1% PBS], CaB [monoclonal, Sigma; diluted 1:200 in 1% PBS-BSA] or beta-III-tubulin (monoclonal, Promega; diluted 1:1000 in 1% PBS-BSA) primary antibodies. After washing the sections with PBS, they were then incubated with (30 min) anti-mouse secondary antibody conjugated to Cys-3, Alexa 488 or Alexa 546 fluorescent dyes (Molecular Probes). The sections were then mounted in the mounting medium. Sections were photomicrographed using Olympus BAX60 Epi-fluorescence Microscope. Images were processed using Adobe Photoshop (version 8.0; San Jose CA, USA).

**Western Blot Analysis**

For immunoblot analysis, frozen hippocampal tissue samples were homogenized in homogenization buffer and centrifuged. The cytoplasmic protein fractions were prepared using cytosol nuclear fractionation kit. The cytosolic fractions with equal amount of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS page) on 4-15% gradient gels. Proteins were transferred to nitrocellulose, blocked for 1 h with blocking solution and incubated for 1 h with either anti-CaB (monoclonal; 1:10,000), or beta-III-tubulin (monoclonal; 1: 1000), antibody. Immunoreactive proteins were visualized by incubation (for 1 h) in the goat antimouse alkaline phosphatase - labeled secondary antibody followed by reaction with the luminescent substrate (Invitrogen). The blots were then exposed to hyperfilm- ECL (Amersham, Arlington Heights, IL, USA).

**RESULTS**

To determine the role of NO in DA induced excitotoxicity, ICR mice were treated with various concentrations of DA in the presence and absence of NOS inhibitors. The effects of DA were qualitatively measured as the appearance of stereotypic neurological signs in the form of scratching. We observed increase in intensity of neurological symptoms with the increasing dosage of DA concentration (Table 1). Animals treated with higher doses of DA (6mg/kg;ip) went into status epilepticus and died within 5-10 min. However, mice pretreated with NOS inhibitors L-NAME and 7-NI showed increased scratching behavior and animals went into status epilepticus even at lower doses of DA (Table 1). On the other hand, pretreatment with NOS precursor L-arginine, stereotypic neurological symptoms were suppressed (Table 1).These data suggest that NOS inhibition
may potentiate DA acid induced effects. However, these alterations may not be caused by direct interaction of DA with NOS as micromolar concentrations of DA did not inhibit NOS activity in vitro (Fig. 1A). In contrast, animals treated with DA and sacrificed after 24 hrs showed a significant loss of nNOS activity (Fig.1B). This may suggest vulnerability of NOS rich neurons to DA. Further, to assess the damage to hippocampal neurons after an acute insult of DA (5mg/kg, ip), cryostat sections were examined for apoptotic cell damage by nick-end labeling of fragmented nuclear DNA. The TUNNEL assay showed a considerable number of apoptotic cells in hilus of dentate gyrus as compared to the control section (Fig 2).

**Table 1:** Effects of Domoic acid (DA) on the home cage behavior of mice. DA was administered in the presence and absence of N-nitro-L-arginine methyl ester (L-NAME) and 7- nitroindazole (7-NI) and L-arginine. DA, L-NAME and L-arginine were mixed in normal saline. 7NI was mixed in DMSO. 4-6 animals were used in each group. L-NAME or 7NI was administered intraperitoneally (ip) 30 min before DA. Mice injected with DA were singly observed for 30-45 min for the occurrence of stereotypic neurological effects in the form of scratching. The animals that went into status epilepticus died within 5-10 min. – no occurrence; + positive signs

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<thead>
<tr>
<th>Treatment and dose (mg/kg i.p.)</th>
<th>Stereotypic neurological effects</th>
<th>Status epilepticus and death</th>
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<td>DA (4) + 7-NI (50)</td>
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- no occurrence, + positive signs, and * one animal went into status epilepticus and recovered

We also determined the long term (30 and 60 days) effects following a single subacute dose of DA. This dose was selected from the dose response experiments. In addition, the animals were subjected to pretreatment with NOS inhibitors. In order to assess if hippocampal damage is progressive, we used various neuronal and glial marker proteins. Immunohistochemistry studies showed loss of calbindin-28k positive neurons in the hippocampus in DA and DA plus L-NAME or 7NI treated animals in both 30 and 60 days treatment regimen (Fig 3). GFAP immunostaining revealed a marked increase in GFAP positive glial cells with large cell bodies and long processes in CA1/2 regions of hippocampus in DA treated animals compared to controls in both 30 and 60 days treatment groups (Fig. 4 and 5). Furthermore, these changes were more pronounced in groups treated with
NOS inhibitors specifically 7NI (Fig 4 and 5). Western bolt analysis also showed a marked decrease in CaB immunoreactive bands in animals treated with NO inhibitor L-NAME as compared to control and DA treated (30 days) animals (Fig. 6). This may indicate a decrease in the levels of CaB in the hippocampus after NOS inhibition.

Figure 1: Effects of DA on nNOS in mouse hippocampus. A: The activity of nNOS prepared from mouse hippocampus was measured in the presence and absence of various concentrations of DA. B: nNOS activity was measured in the hippocampal fractions prepared from mice (n=3) treated with a single dose of 1 and 1.5 mg/kg (ip) DA or vehicle. Animals were sacrificed after 24 hrs. * P<0.05

Figure 2: DNA nick end labeling of hippocampus cryostat sections from control (A) and DA injected (B) mice. Arrows show neurons undergoing apoptosis in hilus of dentate gyrus as compared to the control (A) where no apoptotic cells could be seen.

Figure 3: Calbindin–D28k (CaB) immunoreactivity in the hippocampus. A: Control animal showing CaB D28k positive neurons; B: DA treated animals showing loss of CaB positive neurons after 30 days of treatment; C, D: Sections showing loss of CaB positive neurons in animals treated with DA+L-NAME after 30 and 60 days of treatment respectively; E, F: Sections with loss of CaB immunoreactive neurons in DA+7-NI treated mice after 30 and 60 days of treatment.

Figure 4: GFAP immunoreactivity in CA1/2 region of the mouse hippocampus. A and B: Controls 30 and 60 days group respectively; C and D: DA treated animal showing GFAP positive glial cells; E and F: Large GFAP immunoreactive astrocytes are seen in 30 and 60 days in DA+7NI treated groups respectively. Reactive astrocytes (arrows) show long processes with enlarged cells bodies.
The present study demonstrates that a single sub-acute dose of DA causes a progressive damage to the hippocampus, which is markedly influenced by NOS inhibitors. Further, it establishes that NO may have a protective role in mitigating the toxic effects of DA in a mouse model of temporal lobe epilepsy[9,36,31].

In an epileptic seizure, NO as a retrograde messenger, is believed to initiate a cascade of reactions which prevent the expansion of convulsive activity[2,33]. Both endothelial NOS (eNOS) and neuronal NOS (nNOS) participate in this mechanism. However, there is still some debate as to whether NO is pro- or anti-convulsant. Many studies have shown anti-convulsant effects of NO[26,19,30,18], while others have reported that NO promotes seizure activity[11,25,37], these data may differ depending on dose, administration route and animal species[20].

In an effort to elucidate the role of NO in various seizure models the effects of NOS inhibitors have been extensively studied. In the present study, we found that pretreatment with NOS inhibitors promoted the severity of DA-induced effects in mice. We used L-NAME, a non-selective inhibitors of NOS, which acts on both eNOS and nNOS[19] as well as the relatively selective nNOS inhibitor 7-NI[25,37]. Both L-NAME and 7-NI potentiated DA induced insult. L-NAME treatment seemed to have more generalized inhibitory effect on all forms of NOS as indicated by breathing distress and one animals going into status epilepticus. The animals treated with 7NI, however, had severe neurological symptoms and exhibited more aggressive changes in the hippocampus especially after 30 days post-treatment. Since astrocytes are very sensitive to alterations in the brain environment and respond showing a phenomenon known as astroglial reaction we used increase in GFAP immunoreactivity as a measure of DA induced hippocampal damage. Huttmann and coworkers[15] used transgenic mice with human GFAP promoter-controlled enhanced green fluorescent protein (EGFP) expression. Treatment of these mice with kainate caused a significant increase in the total number of EGFP proliferating radial

**Figure 5:** GFAP immunostained astrocytes in CA1/2 region of the mouse hippocampus in 60-day groups. A: Control; B: DA treated animal showing GFAP positive glial cells; C: Section from DA+L-NAME treated animal showing GFAP reactive astrocytes; D: Immunoreactive astrocytes with characteristically intense GFAP-immunostaining in 60 day DA+7NI treated animal.

**Figure 6:** Western blots of calbindin-D 28k and beta-III-tubulin in the hippocampus in mice in 30 days treatment groups. Equal amounts of protein were loaded onto the 4-15% gels followed by SDS-PAGE, electrophoresis, immunoreaction, and exposure to hyperfilms. Molecular weight markers run in parallel are shown at left. A minimum of three animals per group are shown. A decrease in the intensity of CaB D28k is seen in the treated groups compared to control, but there is no change in the immunointensity of beta-III – tubulin, which was used as a housekeeping protein.

**DISCUSSION**

The present study demonstrates that a single sub-acute dose of DA causes a progressive damage to the hippocampus, which is markedly influenced by NOS inhibitors. Further, it establishes that NO may have a protective role in mitigating...
glia-like astrocytes. Further, a marked decrease in the expression of calcium binding protein CaB, was also assessed. Calbindin was used as marker of neurons that are rich in nNOS\(^{17}\).

The molecular mechanism of NO mediated protection against DA excitotoxicity is not known. However, it has been reported that in kainic acid (KA) model, increased KA-induced seizure activity is accompanied by an increase in cerebral blood flow (CBF), which compensates for local oxygen and glucose demands\(^{20,24}\). A pronounced uncoupling of CBF and seizure induced metabolic demand appears to cause depletion of high energy phosphates and neuronal cell damage. Therefore, extensive inhibition of NOS results in suppression of CBF, increased neuronal cell death during the seizure and enhancement of seizure severity\(^{20}\). It has been demonstrated that eNOS and nNOS derived NO play a major role in the regulation and control of CBF respectively\(^{26}\).

In summary, DA treatment produced both dose- and time-dependent changes in the mouse hippocampus. The effects of a sub-acute dose of DA in short and long term experiments were markedly promoted by NOS inhibitors On the other hand pre-administration of L-arginine suppressed DA induced stereotypic behavior. Our findings in the DA induced animal model of epilepsy thus further support the notion that NO may be involved in the pathogenesis of temporal lobe epilepsy in humans.

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**REFERENCES**

1. Ananth C, Dheen ST, Gopalakrishnakone P, Kaur C. Distribution of NADP H-diaphorase and expression of nNOS, N-methyl-D-aspartate receptor (NMDAR1) and non-NMDA glutamate receptor (GlutR2) genes in the neurons of the hippocampus after domoic acid-induced lesions in adult rats. Hippocampus 2003;13(2):260-72.


