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Anticonvulsant effects of 7-nitroindazole in rodents with reflex epilepsy may result from L-arginine accumulation or a reduction in nitric oxide or L-citrulline formation

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1 To investigate the role of nitric oxide in epilepsy we have studied the effects of agents which affect nitric oxide synthesis in sound-induced seizures in DBA/2 mice and in genetically epilepsy-prone (GEP) rats.

2 The neuronal selective nitric oxide synthase inhibitor, 7-nitroindazole (7-NI) is anticonvulsant in these models with ED_{50} values against clonic seizures in mg kg^{-1} i.p. (times following injection) of: 74 (+0.25 h), 120 (+1 h) in DBA/2 mice, and 56 (+0.25 h), 42 (+0.5 h), 36 (+1 h), 28 (+2 h), 38 (+4 h), 93 (+8 h) in GEP rats.

3 Therapeutic indices (locomotor deficit ED_{50}/anticonvulsant ED_{50}) for 7-NI are low, ranging from 0.6 to 1.1 at +0.25 h to +1 h after administration in GEP rats, but are more favourable at later times (1.6 at +2 h and 2.9 at +4 h).

4 The substrate for nitric oxide synthase, L-arginine (500–5000 mg kg^{-1}, i.p. or 100–300 μg, i.c.v.) but not D-arginine (300 μg i.c.v.) is anticonvulsant in DBA/2 mice. L-Arginine (500–5000 mg kg^{-1}, i.p. or 1800–6000 μg, i.c.v.) is a more potent anticonvulsant than D-arginine (1500–2500 mg kg^{-1}, i.p. or 6000 μg, i.c.v.) in GEP rats.

5 In DBA/2 mice, L-arginine (30 μg i.c.v.) reverses the anticonvulsant effect of 7-NI (50 mg kg^{-1}, i.p.).

6 In GEP rats, low dose L-arginine (25–50 mg kg^{-1}, i.p.) but not D-arginine (50 mg kg^{-1}, i.p.) reverses the anticonvulsant effect of low dose 7-NI (25 mg kg^{-1}, i.p.). A higher dose of L-arginine (500 mg kg^{-1}, i.p.) or 7-NI (50 mg kg^{-1}, i.p.) produces summation of anticonvulsant effect.

7 The product for nitric oxide synthase, L-citrulline (250–831 μg i.c.v.), is convulsant in DBA/2 mice.

8 The anticonvulsant effect of the neuronal selective nitric oxide synthase inhibitor, 7-nitroindazole, may therefore be mediated by L-arginine accumulation, as well as by a reduction in nitric oxide and L-citrulline formation in rodent models of reflex epilepsy.

Keywords: Nitric oxide; arginine; citrulline; 7-nitroindazole; seizures; epilepsy

Introduction

The gaseous chemical messenger, nitric oxide (NO), is thought to be involved in a multitude of physiological processes in the mammalian central nervous system including memory, respiration, and cardiovascular homeostasis (Moncada *et al.*, 1991; Brett & Snyder, 1994; Dawson & Snyder, 1994; Feelisch *et al.*, 1994). NO is formed enzymatically, with L-citrulline as a co-product, by NO synthase from L-arginine and O_2 (with Ca^{2+}, calmodulin and NADPH as co-factors (Knowles *et al.*, 1989; Moncada *et al.*, 1991). Several isoforms of NO synthase have been cloned including neuronal, endothelial and hepatic forms and one form which is present in macrophages (Dawson & Snyder, 1994). The neuronal and endothelial forms of NO synthase are constitutive enzymes dependent upon Ca^{2+} and calmodulin for their activation, whilst NO synthase present in macrophages is inducible and can be activated via Ca^{2+}-independent mechanisms. Thus most NO which is formed in the brain is thought to be derived from the activity of constitutive forms of NO synthase. The pharmacology of NO can be explored with the substrate, L-arginine, and the product, L-citrulline, and with inhibitors of NO synthase, such as various nitro-L-arginine derivatives: N^{ω}-nitro-L-arginine (L-NOARG), N^{ω}-nitro-L-arginine methylester (L-NAME), and N^{ω}-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1990; Moncada *et al.*, 1991; Salter *et al.*, 1995) and nitroindazole derivatives such as 7-nitroindazole (7-NI) and 3-bromo-7-NI (Bland-Ward & Moore, 1995), and with a variety of compounds which facilitate NO formation, such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (SNOG), or 3-morpholinosydnonimine (SIN-1) (Feelisch & Noack, 1987; Moncada *et al.*, 1991), or scavenge NO, such as haemoglobin or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3 oxide (carboxy-PTIO) (Moncada *et al.*, 1991; Maeda *et al.*, 1994). The inhibitors of NO synthase have different selectivity for each form of synthase. The nitroindazole analogues are more selective than the nitro-L-arginine analogues as inhibitors of neuronal than of endothelial NO synthase (Bland-Ward & Moore, 1995).

With regard to pathological conditions, NO has been implicated in animal models of neurodegenerative disease, stroke and epilepsy. Activation of cerebellar NMDA receptors in the rat is thought to lead to the release of NO (Garthwaite *et al.*, 1989). Focal injection of NMDA into the hippocampus of the rat leads to cell death, an effect which can be inhibited by NO synthase inhibitors (Moncada *et al.*, 1992).

In epilepsy, contradictory roles for NO in the development and pathogenesis of seizures have been suggested. Evidence supporting an excitatory role for NO includes findings that L-arginine (300 μg i.c.v.) potentiates NMDA-induced seizures in rats, an effect inhibited by L-NAME (Mollace *et al.*, 1991), and that seizures induced by acetylcholinesterase (AChE) inhibitors and LiCl are inhibited by L-NAME (Bagetta *et al.*, 1992). 7-NI has been shown to reduce kainate-induced NO accumulation and seizures (Mülsch *et al.*, 1994).

Evidence supporting an inhibitory role of NO includes findings that NO has inhibitory feedback effects on the NMDA receptor (Hoyt *et al.*, 1992; Manzoni *et al.*, 1992). L-NOARG results in an enhancement of the kindling process

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(Rondouin *et al.*, 1992), and also increases the severity of kalinic acid (KA)-induced seizures or quinolinate-induced excitotoxicity in rats (Haberney *et al.*, 1992; Penix *et al.*, 1994).

Drawing firm conclusions on the role of NO in epilepsy is complicated since a wide variety of seizure models have been studied and often full dose-responses were not or could not be investigated. In the earlier studies, non-selective inhibitors of NO synthase were employed since they were the most effective inhibitors available, which made the true role of NO in epilepsy difficult to determine.

The discovery of the indazole series of NO synthase inhibitors has transformed the study of NO in the CNS, since the prototype compound, 7-NI, has a greater selectivity as an inhibitor for neuronal NO synthase than for endothelial NO synthase (Babbedge *et al.*, 1993) and has been shown to inhibit rat striatal, cerebellar, hippocampal, cerebral cortex, and olfactory bulb NO synthase activity *in vitro* and *in vivo* (MacKenzie *et al.*, 1994). Unlike L-NOARG, L-NAME or L-NMMA, 7-NI is devoid of hypertensive action in anaesthetized and conscious rodents (Babbedge *et al.*, 1993; Moore *et al.*, 1993a, b; Kelly *et al.*, 1995; Wang *et al.*, 1995).

We have evaluated 7-NI as an anticonvulsant in two animal models of reflex epilepsy. Our colony of genetically-epilepsy prone rats (GEP rats), is a Sprague-Dawley-derived strain from the University of Arizona (Dailey & Jobe, 1985). Wild running, clonic and tonic seizures can be induced repetitively by exposure of the animals to a sound stimulus (10–12 kHz, 60 s, 100–120 dB) with an interval of 1 h or more. A shorter interval of 15 min may be employed for studying compounds with short onset and offset of action (Smith *et al.*, 1993). In contrast to the transiently seizure-susceptible DBA/2 mice, GEP rats remain seizure-susceptible for life, which makes them well-suited for repeated-measure drug studies. In addition, we have determined full dose-response curves and time-course studies for the substrate and product of NO synthase (providing the most comprehensive study yet pertaining to the role of NO in experimental epilepsy). Some of these data have been presented previously in abstract form (Smith *et al.*, 1995; 1996).

Methods

Sound-induced seizures in DBA/2 mice

DBA/2 mice (Institute of Psychiatry colony) of either sex, 3–4 weeks old, weighing between 8–12 g were used. The animals were housed in groups of 6–10 in PVC cages (150 × 300 mm long × 150 mm high) in an environment maintained at 19–22°C and a relative humidity of 55±3% with a 14h/10h light/dark cycle (light on from 0600 to 2000 h). Food and water were available *ad libitum*.

The animals received vehicle or drug treatment ($n=8-21$) and were individually exposed to an audiogenic seizure stimulus (110–120 dB, 12–16 KHz, 60 s) at +0.25 h, +0.5 h, +1 h, +2 h, +4 h, +8 h, and at +24 h after vehicle or drug administration. The incidence of clonic seizure was recorded (Chapman *et al.*, 1984).

Sound-induced seizures in GEP rats

Genetically epilepsy-prone (GEP) rats (Institute of Psychiatry colony) of either sex weighing between 200–350 g were used. The animals were housed in groups of 3–6 in PVC cages (350 × 530 mm long × 180 mm high) in an environment maintained at 19–22°C and a relative humidity of 55±3% with a 14h/10h light/dark cycle (light on from 0600 to 2000 h). Food and water were available *ad libitum*. The animals were individually exposed to an audiogenic seizure stimulus (110–120 dB, 12–16 KHz, 60 s) and the resulting behavioural response was scored on a scale of 0–99: 0 = no response; 1 = wild running; 2 = two episodes of wild running followed by mild clonic seizure; 3 = one episode of wild running followed by mild clonic seizure; 4 = two episodes of wild running followed by severe clonic seizure; 5 = one episode of wild running followed by severe clonic seizure; 6 = two episodes of wild running, clonic seizure followed by incomplete tonic seizure; 7 = one episode of wild running, clonic seizure followed by incomplete tonic seizure; 8 = two episodes of wild running, clonic seizure followed by complete tonic seizure; 9 = one episode of wild running, clonic seizure followed by complete tonic seizure. This was repeated once daily for two more days. Only animals which responded consistently (seizure score = 9) for three consecutive days were employed in the study. On the third day the animals received vehicle or drug treatment ($n=6-12$) and were exposed to a sound stimulus at times designed to determine the onset, peak and offset times of anticonvulsant action for each compound (+0.25, +0.5, +1, +2, +4, +8, +24 h after vehicle or drug administration). No more than three compounds were tested in each animal with a 4 week interval between each new compound studied to allow time for drug wash-out. Previous repeated studies showed this to be an effective method of obtaining reliable ED_{50} values with optimal use of animals (Smith *et al.*, 1993).

Locomotor performance in GEP rats

Groups of 6–8 GEP rats were selected for their ability to remain walking for 60 s on a 9 cm wide rod (with 1.5 mm deep grooves spaced at 10° intervals) revolving at 12 r.p.m. 1 h before drug administration. Further testing was performed at times designed to determine the onset, peak and offset times of locomotor deficit for 7-NI (+0.125 h, +0.25 h, +0.5 h, +1 h, +2 h, +4 h, +8 h, and at +24 h after vehicle or drug administration). Latency to fall (cut-off 60 s) was recorded.

Intracerebroventricular administration of compounds

DBA/2 mice ($n=10$) under halothane anaesthesia received constant depth i.c.v. injections as previously described (Smith & Dürmüller, 1990). The injection volume was 5 μl delivered over 8 s.

GEP rats ($n=8$) were anaesthetized with halothane 2% in N_2O and O_2 and a guide cannula (21 gauge) was placed stereotaxically 2 mm above the left lateral cerebral ventricle (from interaural: AP +8.7, L ±1.3, H +8.0 according to Paxinos & Watson, 1986). The cannula was secured into place with two screws in the cranium and with dental acrylate. The animal recovered from anaesthesia and after 5 days was tested for sound-induced seizures as described above. A thinner 27 gauge i.c.v. cannula, attached with polyethylene tubing to a 25 μl Hamilton syringe, was used to administer vehicle or test compounds to the conscious GEP rat. The injection volume was 5 μl delivered over 90 s. The i.c.v. cannula was left in place for a further 90 s to minimize backflow of the injectate.

Femoral artery cannulation

The left femoral artery was cannulated and the cannula exteriorised via a small skin incision in the area of the dorsal thorax in anaesthetized (2% halothane in 30% O_2 and 70% N_2O) GEP rats. Fifteen minutes after recovery from anaesthesia, mean arterial blood pressure and heart rate were recorded in the conscious GEP rat for 30 min before administration of vehicle or 7-NI (80 mg kg^{-1}, i.p. ($n=5$). Continuous recording of mean arterial blood pressure and heart rate was made in the freely-moving GEP rat for a further 4 h with constant observation. The animals were maintained normothermic with an overhead heating lamp. Food and water were available *ad libitum*.

Chemicals

For i.p. administration, 7-NI(7-nitroindazole m.w. 163.1) (courtesy Dr H.F. Hodson, Wellcome Research Laboratories, Beckenham, U.K.), L-arginine (m.w. 210.7), D-arginine (m.w. 210.7), and L-citrulline (m.w. 175.2) (Sigma Chemical Co., Poole, U.K.) were suspended in 0.25% w/v methylcellulose

(400 centipoise) in h.p.l.c. grade distilled H_2O. The injectate volume in mice for vehicle, 7-NI, and L-arginine was 10 ml kg^{-1}. In rats, the injectate volume for 7-NI, L-arginine or D-arginine was 10 ml kg^{-1}, and for L-citrulline was 10 ml kg^{-1} (for 50–1500 mg kg^{-1}), 20 ml kg^{-1} (for 2500 mg kg^{-1}), or 30 ml kg^{-1} (for 3500–5000 mg kg^{-1}). All compounds were administered at pH = 6.5–7.5.

For i.c.v. administration, the injectate volume was 5 μl. L-arginine, D-arginine or L-citrulline were dissolved in 10 mM phosphate buffered (pH = 7.4) saline (tablet form) (Sigma Chemical Co., Poole, U.K.).

Statistics

Numbers of animals responding per seizure type [wild running (score ≥ 1), clonic (score ≥ 2) and tonic (score ≥ 6) seizures] per experimental group were converted to percentages of those animals responding in the respective vehicle-treated control groups. For DBA/2 mice, only data for clonic seizures are presented. Differences between drug-treated groups were tested for significance against control groups by use of Fisher's Exact test. Latencies to fall(s) as a measure of locomotor performance were converted to percentages of respective vehicle-treated groups. ED_{50} values and 95% confidence limits for the anticonvulsant effects and for the impaired locomotor performance were determined according to the method of Litchfield & Wilcoxon (1949). Cardiovascular data are presented as means ± s.e.mean and were analysed at all time points for each treatment group with respect to the 0 h time point by ANOVA with a Newman Kuels post hoc test.

Results

The anticonvulsant effect of 7-nitroindazole in DBA/2 mice and GEP rats

In DBA/2 mice, 7-NI (64–160 mg kg^{-1}, i.p., $n = 9–21$), but not vehicle, dose-dependently reduced incidence of sound-induced clonic seizures at +0.25 h and at +1 h after administration (Figure 1a). The ED_{50} value (95% confidence limits) against clonic seizures at +0.25 h after administration was 74 (64–87) mg kg^{-1} and at +1 h was 120 (93–154) mg kg^{-1}. A time course study with a sub-maximal anticonvulsant dose of 7-NI (96 mg kg^{-1}, i.p., $n = 8–10$) revealed a peak anticonvulsant effect at +0.125 h to +0.5 h after administration in DBA/2 mice (Figure 1b).

In GEP rats, 7-NI (20–80 mg kg^{-1}, i.p., $n = 6–12$), but not vehicle, dose-dependently reduced the incidence of sound-induced clonic seizures at +0.25 h to +4 h after administration (Figure 1c). The ED_{50} values (95% confidence limits) for reduction of wild running, clonic seizure and tonic seizure at the time of anticonvulsant effect for 7-NI in GEP rats are listed in Table 1).

The effect of 7-NI on locomotor performance in GEP rats

In GEP rats, 7-NI (20–80 mg kg^{-1}, i.p., $n = 7$), but not vehicle, dose-dependently induced locomotor impairment at +0.25 h to +4 h after administration (Figure 1d). The ED_{50} value in mg kg^{-1} (95% confidence limits) for locomotor

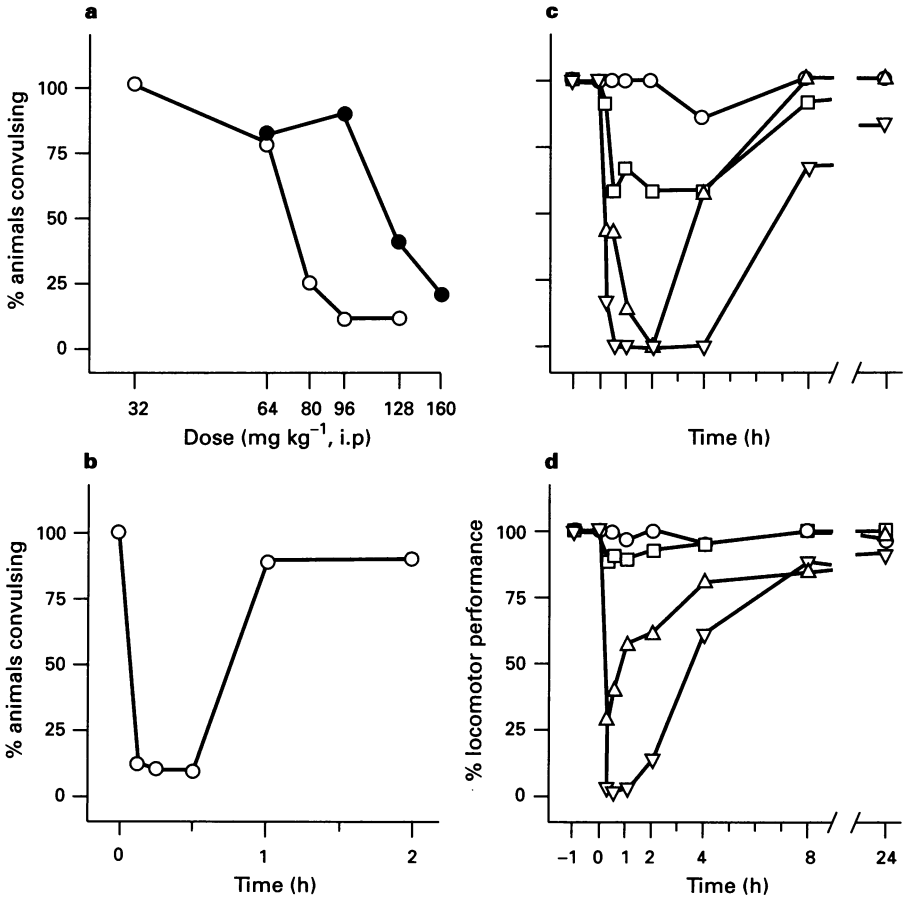

Figure 1 (a) The anticonvulsant effect of 7-nitroindazole 32–160 mg kg^{-1}, i.p. ($n = 9–21$), at (○) + 0.25 h and at (●) + 1 h. (b) The time course of anticonvulsant effect of 7-nitroindazole 96 mg kg^{-1}, i.p. ($n = 8–10$), after administration in DBA/2 mice. (c) The effect of (○) vehicle or 7-nitroindazole (□) 40; (△) 60 and (▽) 80 mg kg^{-1}, i.p. ($n = 6–12$), on sound-induced clonic seizures in GEP rats. Each point is the percentage number of animals showing clonic seizures. (d) The effect of (○) vehicle or 7-nitroindazole (□) 20; (△) 40 and (▽) 80 mg kg^{-1}, i.p. ($n = 7$), on locomotor performance in GEP rats. Each point is the percentage locomotor performance (ability to maintain balance on a rotarod, 100% = 60 s) of a group of 7–8 GEP rats.

Table 1 Anticonvulsant potency of 7-nitroindazole, L-arginine, D-arginine and L-citrulline in genetically epilepsy-prone (GEP) rats

Time (h)	*Wild running*	*Clonic*	*Tonic*
7-Nitroindazole			
0.25	56 (40–79)	56 (40–79)	36 (26–50)
0.5	43 (30–62)	42 (27–63)	21 (14–31)
1	36 (26–50)	36 (26–50)	21 (14–31)
2	28 (18–42)	28 (18–42)	16 (12–22)
4	38 (23–62)	38 (23–62)	22 (15–34)
8	93 (67–130)	93 (67–130)	58 (44–76)
L-Arginine			
0.25	9700 (3919–24006)	6850 (3987–11769)	6850 (3987–11769)
0.5	2433 (1656–3574)	2433 (1656–3574)	2094 (1334–3287)
1	3108 (2083–4636)	3108 (2083–4636)	1905 (1144–3173)
2	2006 (1264–3184)	2006 (1264–3184)	868 (548–1374)
4	1031 (567–1876)	936 (513–1710)	644 (410–1010)
8	1524 (860–2701)	1524 (860–2701)	1059 (656–1706)
24	4820 (2761–8412)	4820 (2761–8412)	4820 (2761–8412)
D-Arginine			
0.25	NH	NH	NH
0.5	NH	NH	NH
1	NH	NH	7271 (1830–28884)
2	2500 (1690–3699)	2500 (1690–3699)	1465 (871–2464)
4	2156 (1555–2987)	1964 (1488–2592)	1353 (825–2219)
8	2034 (1501–2756)	2034 (1501–2756)	1123 (548–2301)
L-Citrulline			
0.25	NH	NH	NH
0.5	NH	NH	NH
1	NH	NH	8598 (4186–17736)
2	10739 (3638–31699)	10739 (3638–31699)	4898 (2500–9597)
4	6109 (3668–10176)	4590 (3066–6870)	2986 (1994–4470)
8	3494 (2080–5868)	3494 (2080–5868)	1023 (328–3188)

Anticonvulsant potency of various agents which may affect NO synthesis in sound-sensitive genetically epilepsy-prone rats. Groups of 6–21 rats received vehicle or 7-nitroindazole (10–80), L-arginine (50–5000), D-arginine (50–5000), L-citrulline (100–5000) (mg kg^{-1}, i.p.) and were exposed to a sound stimulus at +0.25, +0.5, +1, +2, +4, +8, +24 h after vehicle or drug administration. Results are expressed as ED_{50} values in mg kg^{-1} (with 95% confidence limits) for antagonism of wild running, clonic or tonic seizure. NH: Not high enough dose tested.

impairment for 7-NI at +0.25 h was 32 (18–58), at +0.5 h was 36 (19–66), at +1 h was 41 (22–78), at +2 h was 44 (24–80), and at +4 h was 110 (34–356). This resulted in therapeutic indices (ED_{50} value for locomotor deficit/ED_{50} value against clonic seizure) for 7-NI at +0.25 h of 0.6, at +0.5 h of 0.9, at +1 h of 1.1, at +2 h of 1.6, and at +4 h of 2.9.

The cardiovascular effects of 7-nitroindazole in GEP rats

7-NI (80 mg kg^{-1}, i.p., $n=5$) had no significant effect on mean arterial blood pressure (Table 2) but reduced heart rate in GEP rats between 0.5–4 h after administration ($P<0.05$) with a maximal decrease at +1 h of approximately 120 beats min^{-1} (Table 2).

The anticonvulsant effect of L-arginine after i.c.v. administration in DBA/2 mice and GEP rats

L-Arginine (100–300 μg, i.c.v., $n=10$) dose-dependently reduced the incidence of sound-induced clonic seizures in DBA/2 mice at +5 min after administration (Figure 2a). The ED_{50} value (95% confidence limits) against sound-induced clonic seizures at the time of peak effect was 108 (57–203) μg. The onset of anticonvulsant effect of L-arginine (300 μg, i.c.v., $n=10$) was apparent at the earliest test time possible (+5 min) after i.c.v. administration and lasted for 6 h (Figure 2b). D-Arginine (300 μg, i.c.v., $n=10$) was not anticonvulsant in DBA/2 mice (Figure 2a). L-Arginine (1800–6000 μg, i.c.v.) dose-dependently reduced the incidence of sound-induced clonic seizures in GEP rats ($n=8$) at +15 min to +2 h and at +24 h (but not at +48 h; data not shown) after administration (Figure 2c). D-Arginine (6000 μg, i.c.v.) was not anticonvulsant in GEP rats ($n=8$) (Figure 2c).

Table 2 Effect of 7-nitroindazole on mean arterial blood pressure and heart rate in genetically epilepsy-prone (GEP) rats

	Mean arterial blood pressure (mmHg)	
Time (h)	*Vehicle*	*7-NI* (80 mg kg^{-1}, i.p.)
0	117±12	118±2
0.25	121±12	122±2
0.5	118±12	121±3
1	117±10	122±1
2	126±12	126±1
4	127±12	130±3
	Heart rate (beats min^{-1})	
	Vehicle	*7-NI* (80 mg kg^{-1}, i.p.)
0	408±12	416±15
0.25	408±13	375±23
0.5	408±18	321±15
1	412±14	289±9
2	402±22	298±20
4	404±18	297±19

Groups of 5 GEP rats received either vehicle or 7-NI (80 mg kg^{-1}, i.p.) and had mean arterial blood pressure and heart rate recorded for 4 h after vehicle or drug administration. Data are presented as means±s.e.mean.

The anticonvulsant effect of L-arginine after i.p. administration in DBA/2 mice and GEP rats

In DBA/2 mice, L-arginine (500–5000 mg kg^{-1}, i.p., $n=10$), but not vehicle, dose-dependently reduced the incidence of sound-induced clonic seizures at +0.25 h and at +1 h after administration. The ED_{50} value against clonic seizures at

+0.25 h after administration was 2077 (888–4858) mg kg^{-1} and at +1 h was 8800 (1581–48991) mg kg^{-1} (i.p.)

In GEP rats, L-arginine (500–1500 mg kg^{-1}) had more potent anticonvulsant effects when compared with equimolar doses of D-arginine (500–1500 mg kg^{-1}), or L-citrulline (416–1247 mg kg^{-1}) (i.p. $n=6-12$) (Figure 3a-c). The onset of anticonvulsant action was slower for D-arginine (2500 mg kg^{-1}) than for L-arginine (2500 mg kg^{-1}), although the maximum effect was the same (Figure 3d). L-Arginine (5000 mg kg^{-1}) was fully anticonvulsant, while GEP rats which received D-arginine (5000 mg kg^{-1}) died. The ED_{50} values for reduction of wild running, clonic seizure and tonic seizure at the time of anticonvulsant effect for L-arginine, D-arginine and L-citrulline (i.p.) in GEP rats are listed in Table 1.

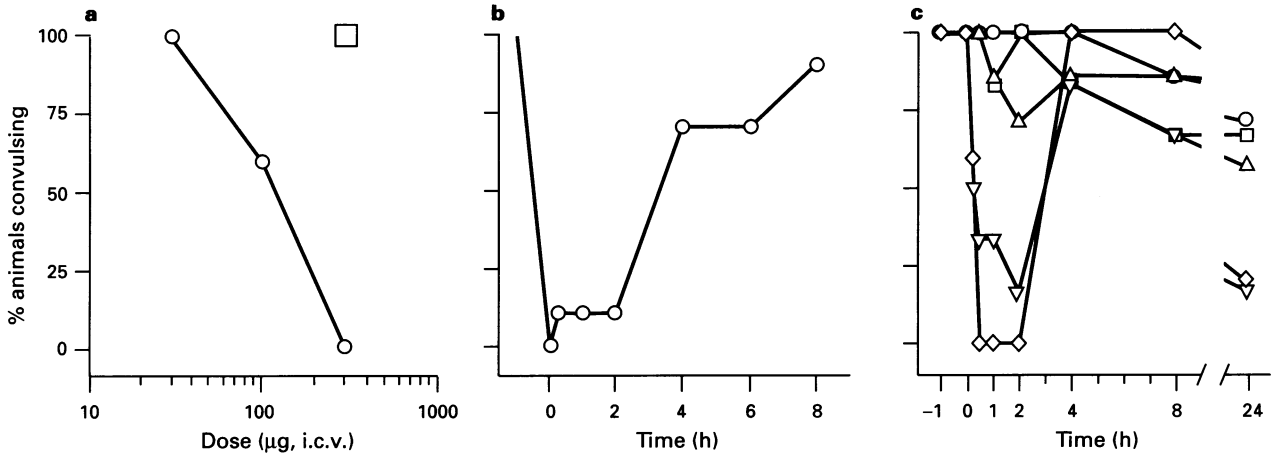

Figure 2 (a) The effect of (○) L-arginine 30–300 μg, i.c.v., or (□) D-arginine 300 μg, i.c.v., on sound-induced clonic seizures in DBA/2 mice. (b) The time course of the anticonvulsant effect of L-arginine 300 μg, i.c.v., in DBA/2 mice. (c) The effect of (○) vehicle or (□) D-arginine 6000 μg or L-arginine (△) 1800; (▽) 3000 and (◇) 6000 μg, i.c.v., on sound-induced clonic seizures in GEP rats. Each point is the percentage number of 7–10 animals showing clonic seizures.

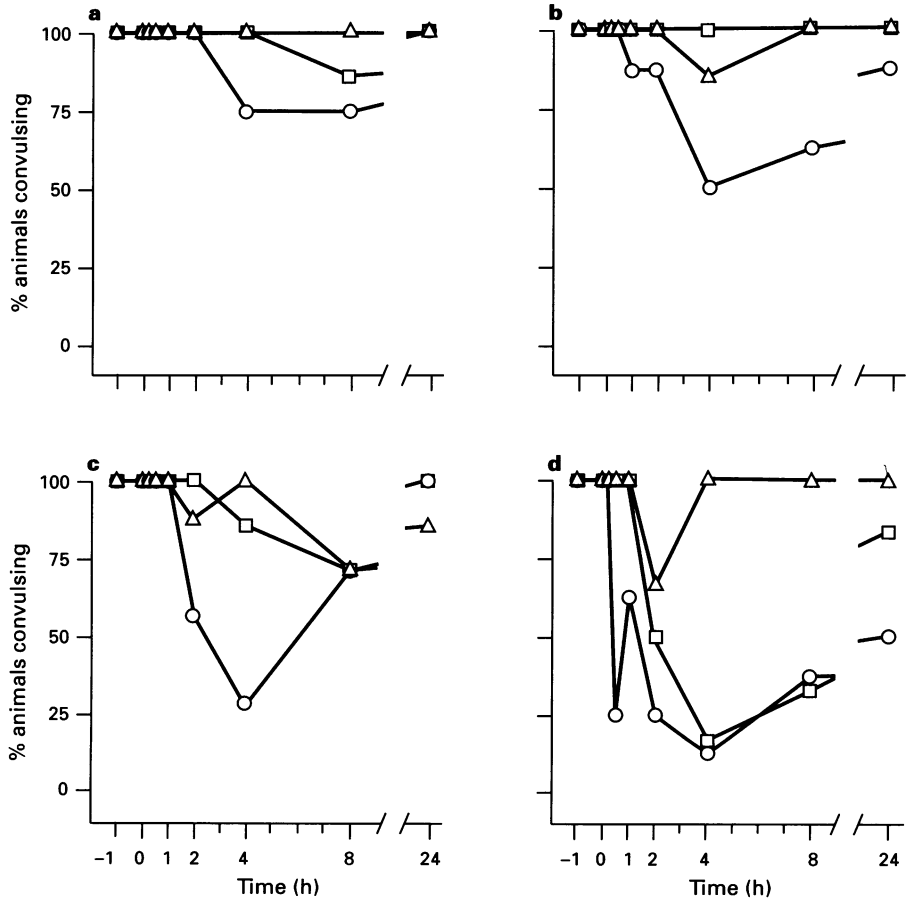

Figure 3 The effect of (○) L-arginine, (□) D-arginine or equimolar doses of (△) L-citrulline: (a) 500 mg kg^{-1}, (b) 1000 mg kg^{-1}, (c) 1500 mg kg^{-1}, (d) 2500 mg kg^{-1}, (i.p.) on sound-induced clonic seizures in GEP rats. Each point is the percentage number of 6–12 animals showing clonic seizures.

Reversal of the anticonvulsant effect of 7-nitroindazole with L-arginine in DBA/2 mice and GEP rats

To test whether we could reverse the anticonvulsant effect of 7-NI, DBA/2 mice received co-injection of L-arginine (160–500 mg kg^{-1}) (doses which themselves had no anticonvulsant effect) and 7-NI (80 mg kg^{-1}) (i.p., $n=10$), or a combination of 7-NI (50–80 mg kg^{-1}) 10 min before L-arginine or D-arginine (30 μg i.c.v., $n=10-20$) (a dose which had no anticonvulsant effect) and exposed to an audiogenic stimulus at +0.25 h after vehicle or 7-NI administration.

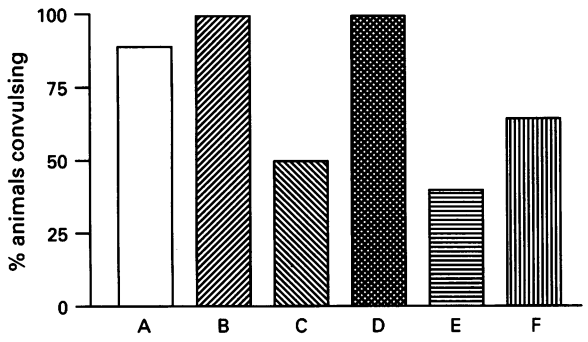

Figure 4 The effect of vehicle/vehicle (A); vehicle/L-arginine, 30 μg (B); 7-nitroindazole (7-NI, 50 mg kg^{-1})/vehicle (C); 7-NI, 50 mg kg^{-1}/L-arginine, 30 μg (D); 7-NI, 80 mg kg^{-1}/vehicle (E); 7-NI, 80 mg kg^{-1}/L-arginine, 30 μg (15 min pretreatment i.p./5 min pretreatment i.c.v.) (F) on sound-induced clonic seizures in DBA/2 mice. Each column is the percentage number of 10–20 DBA/2 mice showing clonic seizures.

In DBA/2 mice, co-administration of L-arginine (160–500 mg kg^{-1}, i.p.) had no significant effect on the anticonvulsant effect of 7-NI (80 mg kg^{-1}, i.p.) at +0.25 h (data not shown).

L-arginine (30 μg, i.c.v., −5 min) significantly reversed the anticonvulsant effect of 7-NI (50 mg kg^{-1}, i.p., −15 min) (Figure 4).

To test whether we could reverse the anticonvulsant effect of 7-NI with arginine, GEP rats received vehicle or a co-injection of 7-NI (25 or 50 mg kg^{-1})/L-arginine (25, 50 or 500 mg kg^{-1}), or a co-injection of vehicle or 7-NI (25 mg kg^{-1})/D-arginine (50 mg kg^{-1}) (i.p., $n=8-32$) and exposed to an audiogenic stimulus at +0.25 h, +0.5 h, +1 h, +2 h, +4 h, +8 h, +24 h after administration.

L-arginine (25 mg kg^{-1}) apparently reversed the anticonvulsant effect of 7-NI (25 mg kg^{-1}) in GEP rats at +1 h after systemic administration although this failed to reach statistical significance (Figure 5a). L-Arginine (50 mg kg^{-1}, i.p.) significantly reversed the anticonvulsant effect of 7-NI (25 mg kg^{-1}, i.p.) in GEP rats at +1 h after systemic administration ($P<0.05$, Fisher's Exact test) (Figure 5b). The anticonvulsant effect of a higher dose of 7-NI, 50 mg kg^{-1}, i.p., was slightly potentiated by L-arginine (50 mg kg^{-1}, i.p.) although this failed to reach statistical significance (Figure 5c). L-Arginine (500 mg kg^{-1}, i.p.) had no effect on the anti-convulsant effect of 7-NI (50 mg kg^{-1}, i.p.) (Figure 5d). D-Arginine (50 mg kg^{-1}, i.p.) had no effect on the anticonvulsant effect of 7-NI (25 mg kg^{-1}, i.p.) (data not shown).

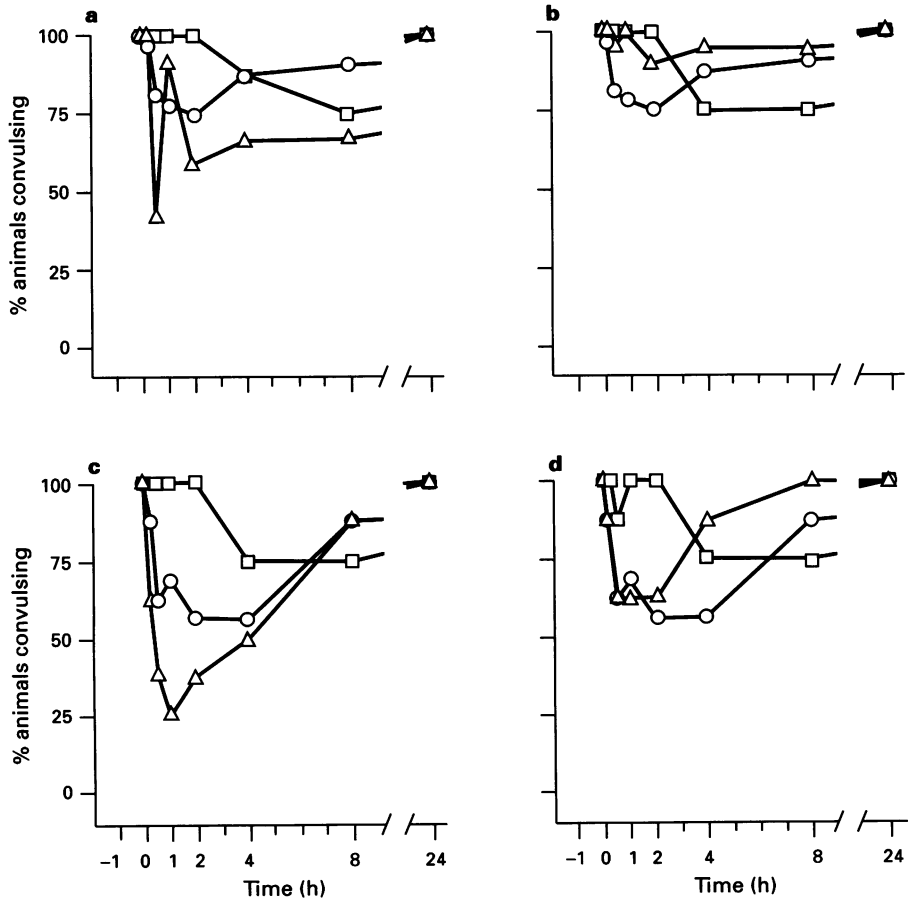

Figure 5 The effect of coadministration of (a) (○) 7-nitroindazole (7-NI), 25 mg kg^{-1}/vehicle (veh); (□) veh/L-arginine (L-arg), 25 mg kg^{-1}; (△) 7-NI, 25 mg kg^{-1}/L-arg, 25 mg kg^{-1}; (b) (○) 7-NI, 25 mg kg^{-1}/veh; (□) veh/L-arg, 50 mg kg^{-1}; (△) 7-NI, 25 mg kg^{-1}/L-arg, 50 mg kg^{-1}; (c) (○) 7-NI, 50 mg kg^{-1}/veh; (□) veh/L-arg 50 mg kg^{-1}; (△) 7-NI, 50 mg kg^{-1}/L-arg 50 mg kg^{-1}; (d) (○) 7-NI, 50 mg kg^{-1}/veh; (□) veh/L-arg, 500 mg kg^{-1}; (△) 7-NI, 50 mg kg^{-1}/L-arg, 500 mg kg^{-1} on sound-induced clonic seizures in GEP rats. Each point is the percentage number of 8–32 GEP rats showing clonic seizures.

Convulsant effects of L-citrulline in DBA/2 mice and GEP rats

L-Citrulline (250–831 μg, i.c.v., $n=10$) dose-dependently induced seizures in DBA/2 mice within 5–15 min after administration. The dose that caused clonic seizures in 50% of animals (95% confidence limits) was 274 (30–2422) μg.

L-Citrulline (42–2079 mg kg^{-1}, i.p.) did not have anticonvulsant effects in GEP rats (Figure 3a–d). L-Citrulline 2910 mg kg^{-1}, i.p., showed slight anticonvulsant effect and L-citrulline 4158 mg kg^{-1}, i.p., reduced the incidence of sound-induced clonic seizures to 17% at +8 h after drug administration enabling ED_{50} values against sound-induced seizures to be determined (Table 1).

Adverse behavioural effects

In DBA/2 mice, 7-nitroindazole 160 mg kg^{-1}, i.p., induced severe ataxia with loss of righting reflex. L-Arginine 5000 mg kg^{-1}, i.p., induced slight ataxia without loss of righting reflex between 0.25–1 h, and a significant (2–4°C) decrease in rectal temperature. The rectal temperature of the animals was maintained normothermic by use of an overhead heating lamp. L-Arginine (50 g kg^{-1}, i.p.) caused severe ataxia with loss of righting reflex and myoclonus within 2 min after administration. L-Arginine (300 μg, i.c.v.) induced behavioural excitation including head bobbing, increased locomotion, and circling between 5–10 min after administration. These effects were absent after 30 min. In GEP rats, 7-nitroindazole (80 mg kg^{-1}, i.p.), L-arginine (5000 mg kg^{-1}, i.p.), and D-arginine (2500 mg kg^{-1}, i.p.) induced severe ataxia with loss of righting reflex from 0.5–8 h after drug administration. 7-Nitroindazole (40 mg kg^{-1}, i.p.) and L-arginine (2500 mg kg^{-1}, i.p.) induced slight ataxia

Discussion

We have shown that 7-nitroindazole is anticonvulsant in DBA/2 mice and GEP rats with ED_{50} values against clonic seizures (at the time of peak effect) of 74 mg kg^{-1} (+0.25 h), and 28 mg kg^{-1} (+2 h), respectively. A fully anticonvulsant dose of 7-NI (80 mg kg^{-1}, i.p.) had no effect on blood pressure, but caused a decrease in heart rate by approximately 120 beats min^{-1} (30%) and induced severe ataxia between 1–4 h after administration in conscious GEP rats. The therapeutic index (ED_{50} value for locomotor deficit/ED_{50} value for antagonism of clonic seizure) for 7-NI in GEP rats was low ranging from, 0.6–2.9, although low therapeutic indices have been obtained for conventional anticonvulsants in the GEP rat model of reflex epilepsy (Smith *et al.*, 1993). An apparent anticonvulsant effect of 7-NI (25–100 mg kg^{-1}, i.p.) has recently been described against pilocarpine-induced seizures in mice (Van Leeuwen *et al.*, 1995). This effect was not reversed by pretreatment with L-arginine (300 mg kg^{-1}, i.p.).

L-Arginine was anticonvulsant after i.p. or i.c.v. administration in DBA/2 mice or GEP rats. This anticonvulsant effect was found to be enantioselective in that only high doses of D-arginine (1500–2500 mg kg^{-1}, i.p.) which showed toxicity (severe ataxia) were anticonvulsant in GEP rats. L-Arginine, 300 μg, i.c.v., induced excitatory activity (hyperlocomotion and circling) in DBA/2 mice similar to that described by Mollace *et al.* (1991) in rats. Nevertheless, we found L-arginine, 300 μg i.c.v., to be anticonvulsant against sound-induced seizures in DBA/2 mice and GEP rats at a time when it reversed the anticonvulsant effect of L-NAME against NMDA-induced seizures in rats (Mollace *et al.*, 1991). Accumulation of L-arginine in the central nervous system may therefore be a mechanism which contributes to the anticonvulsant effect of the NO synthase inhibitor, 7-NI. More consistent with the findings of Mollace & co-workers (1991), we found that a very high dose of L-arginine (1000 μg, i.c.v.) was convulsant in DBA/2 mice (Smith *et al.*, 1996). The mechanism of anticonvulsant action of L-arginine is unknown, but is enantioselective. There is increasing evidence that D-amino acids can be converted to L-amino acids via the action of a D-amino acid oxidase coupled with transamination (D'Aniello *et al.*, 1993; Horiike *et al.*, 1994) which may explain the anticonvulsant effect of high dose D-arginine in this study and lack of enantioselective effect of arginine in other studies (see Smith, 1992). L-Arginine may have a direct anticonvulsant action or a product of its metabolism may be anticonvulsant. Possible products include ornithine via the urea cycle, citrulline and NO by NO synthase (Dawson & Snyder, 1994) and agmatine by arginine decarboxylase (Li *et al.*, 1995). In macrophages, L-citrulline is a weak ($K_i=3.4$ mM) but more potent inhibitor of L-arginine uptake than L-arginine is an inhibitor of L-citrulline uptake. Some conversion of L-citrulline to L-arginine is possible under conditions of limited L-arginine availability (Baydoun *et al.*, 1994). Uptake of L-arginine could be an important regulatory step in the pathway of NO formation (Lopes *et al.*, 1994). However, an anticonvulsant effect mediated by effects on substrate or product uptake is unlikely since in the present study L-arginine had anticonvulsant effects immediately after i.c.v. administration, a route which should mainly avoid carrier-mediated effects at least from the vasculature to the cerebrospinal fluid. Until structure-activity studies with arginine as the lead compound are undertaken we therefore remain uncertain of the precise mechanism of the anticonvulsant effect of arginine in reflex epilepsy.

Since high doses of L-arginine are anticonvulsant, the anticonvulsant effect of 7-NI against sound-induced seizures was reversed by L-arginine only in limited conditions. In DBA/2 mice, administration of L-arginine 30 μg, i.c.v., but not L-arginine, 160 mg kg^{-1}, i.p., reversed the anticonvulsant effect of 7-NI 50 mg kg^{-1}, i.p., at +0.25 h after administration.

In GEP rats, a reversal of the anticonvulsant effect of 7-NI with L-arginine was more readily demonstrable since the full time course of anticonvulsant effect was analysed. A reversal of the anticonvulsant effect of a low dose of 7-NI (25 mg kg^{-1}, i.p.) was achieved with a low dose of L-arginine (50 mg kg^{-1}, i.p.) at +1 h after administration. As with the anticonvulsant effect of L-arginine (i.p. or i.c.v.) this reversal was enantioselective in that D-arginine had no effect on the anticonvulsant effect of 7-NI. At durations greater than +1 h after administration the anticonvulsant effect of low dose 7-NI (25 mg kg^{-1}, i.p.) was absent and therefore could not be reversed. When the same dose of L-arginine (50 mg kg^{-1}, i.p.) was combined with a higher dose of 7-NI (50 mg kg^{-1}, i.p.) the reversal of the anticonvulsant effect of 7-NI was lost. 7-NI interacts with the arginine site of NO synthase in a competitive manner (Babbedge *et al.*, 1993). Thus when co-administered with a low dose of 7-NI, L-arginine may reverse the anticonvulsant effect of 7-NI, (its conversion to L-citrulline and NO maintaining its concentration below a threshold concentration for anticonvulsant effect). When the dose of 7-NI is increased, there is a greater inhibition of NO synthase and a second mechanism may prevail, namely, the concentration of L-arginine may increase to a level which is anticonvulsant in genetically-epilepsy prone animals.

There have been numerous studies showing that NO synthase inhibitors are convulsant in rodents. L-NOARG results in an enhancement of the kindling process (Rondouin *et al.*, 1992; 1993) and can enhance the severity of kindled seizures in rats (Herberg *et al.*, 1995). L-NOARG or L-NAME increase the severity of KA-induced or bicuculline-induced seizures or quinolinate-induced excitotoxicity in rats (Haberney *et al.*, 1992; Penix *et al.*, 1994; Przegalinski *et al.*, 1994; Wang *et al.*, 1994; Maggio *et al.*, 1995) or have little effect (L-NAME) (Bagetta *et al.*, 1995). NO has inhibitory feedback effects on the NMDA receptor (Hoyt *et al.*, 1992; Manzoni *et al.*, 1992). These data have been interpreted as supporting an anticonvulsant role for NO in epilepsy, but their interpretation is complicated by the variety of effects of non-selective inhibitors of NO synthase. Although, L-NAME (20 mg kg^{-1}) decreases hippocampal extracellular glutamate concentration (which

might contribute to an anticonvulsant effect) during KA-induced seizures in rats, there is increased severity of seizures accompanied by decreased hippocampal blood flow preceeding mortality (Rigaud-Monet *et al.*, 1994; 1995) consistent with the findings of Haberney *et al.* (1992), Penix *et al.* (1994), and Maggio *et al.* (1995). Furthermore, focal administration of bicuculline induces cortical hyperaemia accompanying seizures which are inhibited by L-NOARG, but seizure propensity is unaffected (Pereira-de-Vasconcelos *et al.*, 1995), suggesting that NO mediates vascular responses during seizures, although results with systemic administration of L-NOARG are less supportive of this hypothesis (Wang *et al.*, 1994; Theard *et al.*, 1995)

Many results, however, support a convulsant role for NO in epilepsy: kindling produces a long-lasting increase in NO synthase activity (Al-Ghoul *et al.*, 1995). Direct administration of NO 330–800 μmol into the brain of rats has been attempted resulting in brief tonic convulsive episodes (Smith *et al.*, 1991). L-Arginine (300 μg i.c.v.) potentiates NMDA-induced seizures in rats, an effect inhibited by L-NAME (Mollace *et al.*, 1991). Seizures induced by AChE inhibitors and LiCl, cocaine, pentylenetetrazol or bicuculline have been found to be inhibited by L-NOARG, L-NAME or L-NMMA (Bagetta *et al.*, 1992; Osonoe *et al.*, 1994; Przewlocka *et al.*, 1994; Hara *et al.*, 1996). The problem of utilizing non-selective inhibitors of NO in CNS studies is demonstrated in the studies of Rundefeldt and coworkers, in that the threshold for seizures after cortical electrical stimulation in rats was increased by L-NOARG (1–10 mg kg^{-1}, i.p.), but L-NOARG (40 mg kg^{-1}, i.p.) decreased the threshold for seizures (Rundfeldt *et al.*, 1995). Unlike L-NOARG, L-NAME or L-NMMA, 7-NI has been shown to be devoid of hypertensive action in anaesthetized and conscious rodents (Babbedge *et al.*, 1993; Moore *et al.*, 1993a, b; Kelly *et al.*, 1995; Wang *et al.*, 1995). 7-NI reduces kainate-induced NO accumulation (assessed by spin-trapping) and seizures in rats (Mülsch *et al.*, 1994) and reduces seizures induced by pilocarpine in mice (Van Leeuwen *et al.*, 1995). Our data are consistent with these results with 7-NI in chemically-induced seizure models and support a convulsant role of NO generated by neuronal NO synthase.

Many workers have postulated that reversal of any proconvulsant effect of NO synthase inhibitors by L-arginine argues for an anticonvulsant role for NO in epilepsy. The role of the co-product of NO synthase, L-citrulline, in epilepsy has been ignored in those studies. However, as we have shown in DBA/2 mice in this study, a convulsant role in genetically epilepsy-prone animals can be ascribed to L-citrulline. When given systemically, L-citrulline has no convulsant effect and only high doses which show toxicity are anticonvulsant. We have also shown in preliminary studies that the NO donor, SIN-1, is convulsant in DBA/2 mice and GEP rats (Smith *et al.*, 1996). Our results therefore suggest that the NO synthase inhibitor, 7-NI, is anticonvulsant by potentially blocking the formation of two endogenous convulsant agents, NO and L-citrulline.

In this study we have shown that the neuronal selective NO synthase inhibitor, 7-NI, is anticonvulsant after systemic administration in two rodent models of reflex epilepsy. Studies with 7-NI in other epilepsy models will provide more meaningful results than those obtained with inhibitors of the vascular form of NO synthase.

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