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A role of nitric oxide in organophosphate-induced convulsions

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Abstract

The effects of nitric oxide-regulating compounds on convulsions and mortality of rats administered i.p. with diisopropylfluorophosphate was investigated. L-N^G-nitroarginine methyl ester, a nitric oxide synthase inhibitor possessing an anticholinergic action, markedly attenuated the intensity of convulsions and significantly reduced the mortality rate. A similar result was obtained with anticholinergic procyclidine, an *N*-methyl-D-aspartate receptor antagonist. Noteworthy, L-N^G-nitroarginine, another inhibitor of nitric oxide synthase, significantly attenuated the seizure intensity when administered in combination with atropine sulfate (5 mg/kg), though either L-N^G-nitroarginine or atropine sulfate was inactive alone. It is suggested that nitric oxide may be a proconvulsant or a convulsion-promoting factor in anticholinesterase poisoning, and both the reduction of nitric oxide level and blockade of cholinergic systems may be required for more effective protection of seizures. © 1997 Elsevier Science B.V.

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1. Introduction

Recently, interest in the prevention of neuronal damage induced by seizures has increased (Upton, 1994; Gerlach et al., 1995). It has been demonstrated that excitatory amino acids and their agonists could be toxic to central nervous tissues (Garthwaite and Garthwaite, 1989), and might play an important role in neurological disorders such as stroke, epilepsy and chronic neurodegenerative diseases (Upton, 1994; Gerlach et al., 1995). It was reported that excitatory amino acids increased during convulsions following organophosphate intoxication (Lallement et al., 1991). Accordingly, antagonists of excitatory amino acid receptors, especially *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor, are expected to be potentially useful to prevent convulsions and neuronal damage induced by organophosphate poisoning (Shih et al., 1991).

In the central nervous system (CNS), the activation of nitric oxide synthase following stimulation of the NMDA receptor, produces nitric oxide which functions as a signalling or cytotoxic molecule (Dawson et al., 1991). As a retrograde messenger, nitric oxide induces the release of several neurotransmitters including excitatory amino acid L-glutamate (Montague et al., 1994), which deranges neurotransmitter balance and affects neuronal excitability. Meanwhile, nitric oxide also induces feedback antagonism of NMDA receptors via its redox-modulatory action (Lei et al., 1992) or other mechanims (Hoyt et al., 1992), which may protect them from sustained overstimulation (Schuman and Madison, 1994). Interestingly, it was reported that nitric oxide regulates excitatory amino acid release in a biphasic manner according to the level of its production in vivo (Segieth et al., 1995). This may be one of the reasons why conflicting results are yielded with nitric oxide synthase inhibitors; L-N^G-nitroarginine methyl ester (L-NAME) and L-N^G-monomethyl arginine (L-NMMA) attenuated pentylenetetrazol or kainate-in-

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duced seizures (Osonoe et al., 1994; Przegalinski et al., 1994), while quinolinate or kainate-induced seizures were potentiated by L-NAME and L-N^G-nitroarginine (L-NA) (Haberny et al., 1992; Rondouin et al., 1993). Moreover, L-NAME exerted controversial results in cholinergic models of epilepsy (Bagetta et al., 1992; Starr and Starr, 1993). Thus, it was suggested that nitric oxide played a role as a proconvulsant as well as an anticonvulsant molecule. This led us to investigate the possible role of nitric oxide in the convulsions induced by organophosphate poisoning. For this purpose, nitric oxide synthase inhibitors, procyclidine hydrochloride, an NMDA receptor antagonist (Olney et al., 1987; McDonough and Shih, 1995), and L-arginine methyl ester (L-AME), a precursor of nitric oxide, were tested to support the evidences of involvement of nitric oxide in diisopropylfluorophosphate (DFP)-induced convulsions.

2. Materials and methods

The experimental protocols were designed to induce maximal mortality following severe convulsions and to properly evaluate the effects of candidate compounds. Adult Sprague-Dawley female rats (200–300 g) were poisoned with a low (6 mg/kg, 1.2 LD_{50}) or a high (10 mg/kg, 2.0 LD_{50}) dose of DFP. In a low-dose poisoning, each candidate compound was administered i.p. 30 min prior to i.p. challenge with DFP. Separately, each compound was treated in combination with atropine sulfate (15 mg/kg) which was injected i.m. 10 min before DFP poisoning. In a high-dose poisoning, pyridostigmine bromide and atropine methylnitrate, which are centrally inactive (Shih et al., 1991), were pretreated to reduce the mortality and eliminate peripheral signs. This combination might be preferable to that of atropine methylnitrate and 1-[([4-(aminocarbonyl)-pyridinio]methoxy)methyl]-2-[(hydroxyimino)-methyl]-pyridinium dichloride monohydrate (HI-6) previously used in soman (2.0 LD_{50}) poisoning (Tryphonas and Clement, 1995), since HI-6 was reported to exert CNS effects. If otherwise described, pyridostigmine bromide (0.1 mg/kg) and atropine methylnitrate (20 mg/kg) were injected i.m. 30 min and 10 min before DFP poisoning, respectively. The intensity of convulsions was evaluated using 5-point scores (De Sarro et al., 1993) as follows; 0, no response; 1, myoclonic jerks of the contralateral forelimb; 2, mouth and facial movements (i.e., facial myoclonus, clonus of the jaw and vibrissae) and head nodding with or without mild forelimb clonus; 3, severe forelimb clonus; 4, rearing and severe forelimb clonus; 5, rearing and falling. All compounds were dissolved in saline, except DFP which was diluted in distilled water. DFP, pyridostigmine bromide, atropine sulfate and atropine methylnitrate were administered in a volume of 1 ml/kg, and candidate drugs were injected in a volume of 5 ml/kg.

Results were expressed as the mean ± S.D. of six determinations. Tests of significance were performed using unpaired Student's t-test, with $P < 0.05$ as a criterion of difference.

3. Results

Intraperitoneal administration of DFP (6 mg/kg, 1.2 LD_{50}) to rats induced severe convulsions (mean score 4.1) concomitant with cholinergic signs such as muscle fasciculations, tremor and salivation, which started 6 min after poisoning, leading to 100% mortality of animals within 24 h (Table 1). Atropine sulfate (15 mg/kg) delayed the onset of convulsions to some extent. A similar result was obtained with an anticholinergic compound, procyclidine hydrochloride (10–30 mg/kg) expressing an NMDA receptor antagonism. Procyclidine in combination with atropine sulfate exerted an enhanced protection. Interestingly, L-NAME, a nitric oxide synthase inhibitor possessing an anticholinergic action, significantly delayed the onset of convulsions, and attenuated the intensity of symptoms in a dose-dependent manner, resulting in complete survivability. In combination with atropine sulfate, L-NAME (100 mg/kg) eliminated the symptoms almost completely, whereas L-AME did not exhibit any considerable effect.

Another set of experiments were performed using a higher dose (10 mg/kg, 2.0 LD_{50}) of DFP in rats pretreated with centrally-inactive pyridostigmine and atropine methylnitrate to reduce the lethality. This system successfully eliminated peripheral signs, and demonstrated the seizure activity more clearly. A higher dose of DFP to rats, pretreated with pyridostigmine and atropine methylnitrate, induced typical central symptoms (Table 2) such as severe forelimb clonus and head nodding (mean score 3.2). Such symptoms were significantly attenuated by treatment with procyclidine (30 mg/kg). Similarly, a good protection was obtained with L-NAME (100 mg/kg), but neither L-NA (30 mg/kg), another potent nitric oxide synthase inhibitor lacking in anticholinergic action, nor L-AME (100 mg/kg). Rather, L-AME significantly potentiated the seizure intensity (mean score 4.4). These results suggest the possible involvement of nitric oxide in epileptogenesis by a high dose of DFP. Noteworthy, L-NA (30 mg/kg) in combination with atropine sulfate at a low dose (5 mg/kg) significantly attenuated the seizure intensity (mean score 0.9), though neither were effective when administered alone.

Table 1
Effect of candidate compounds, alone or in combination with atropine sulfate ($AtSO_4$), on diisopropylfluorophosphate (DFP) poisoning

Treatment (mg/kg)	Covulsions		Mortality (%)
	Time-to-onset (min)	Intensity	
DFP alone (6)	6.0 ± 2.2	4.1 ± 0.7	100
+ $AtSO_4$ (15)	11.3 ± 4.3*	3.1 ± 1.3	40
+ PC (10)	9.5 ± 2.8*	2.7 ± 1.1*	40
+ PC (30)	10.5 ± 3.5*	2.0 ± 0.5*	0
+ L-NAME (30)	18.0 ± 6.1*	2.3 ± 0.6*	0
+ L-NAME (100)	22.0 ± 5.7*	1.2 ± 0.5*	0
+ L-AME (100)	6.8 ± 3.0	4.3 ± 0.8	70
+ $AtSO_4$ (15) + PC (10)	23.5 ± 7.1**;*	1.0 ± 0.5**;*	0
+ $AtSO_4$ (15) + PC (30)	ND	0.5 ± 0.5**;*	0
+ $AtSO_4$ (15) + L-NAME (30)	ND	0.5 ± 0.4**;*	0
+ $AtSO_4$ (15) + L-NAME (100)	ND	0.1 ± 0.3**;*	0
+ $AtSO_4$ (15) + L-AME (100)	15.6 ± 4.6*	3.3 ± 1.0	30

Each compound was administered i.p. 30 min prior to i.p. challenge with 6.0 mg/kg (1.2 LD_{50}) of DFP ($n = 6$). $AtSO_4$ was injected i.m. 10 min before DFP poisoning. All compounds were dissolved in saline, except DFP which was diluted in distilled water.
PC, procyclidine hydrochloride; L-NAME, L-N^G-nitroarginine methyl ester; L-AME, L-arginine methyl ester; ND, not determined.
* Significantly different from control (DFP alone) ($P < 0.05$).
** Significantly different from $AtSO_4$ ($P < 0.05$).

4. Discussion

Poisoning with organophosphate soman (pinacolylmethyl phosphonofluoridate), a potent inhibitor of cholinesterases, has induced elevation of excitatory amino acids in CNS, prolonged limbic seizures and subsequent neuropathy (McDonough et al., 1989; Lallement et al., 1991). Anticonvulsant benzodiazepines which are *r*-aminobutyric acid (GABA) receptor agonists have been used for blocking convulsions and neuronal damage induced by soman poisoning (McDonough et al., 1989; Shih et al., 1991). In addition, antagonists of NMDA receptors act as anticonvulsants in soman-induced seizures, and thereby prevent neuronal damage (McDonough et al., 1989; Shih et al., 1991). These results imply that diazepam antagonizes NMDA receptors, which is supported by a recent observation that diazepam, a benzodiazepine, protected mice from NMDA-induced lethality with an ED_{50} value of 0.1 mg/kg (McDonough and Shih, 1995).

Table 2
Effect of candidate compounds in combination with pyridostigmine bromide (PS) and atropine methylnitrate (AtMN) on diisopropylfluorophosphate (DFP) poisoning

Treatment (mg/kg)	Seizure intensity
PS + AtMN alone	3.1 ± 0.7
+ PC (30)	0.5 ± 0.3*
+ L-NAME (100)	0.4 ± 0.3*
+ L-AME (100)	4.4 ± 0.6*
+ L-NA (30)	3.4 ± 0.6
+ L-NA (30) + $AtSO_4$ (5)	0.9 ± 0.5*
+ $AtSO_4$ (5)	2.8 ± 0.8

Each candidate compound was administered i.p. 30 min prior to the i.p. challenge with 10 mg/kg (2.0LD_{50}) of DFP ($n = 6$). PS (0.1 mg/kg) and AtMN (20 mg/kg) were injected i.m. 30 min and 10 min before DFP poisoning, respectively. All compounds were dissolved in saline, except DFP which was diluted in distilled water.
PC, procyclidine hydrochloride; L-NAME, L-N^G-nitroarginine methyl ester; L-AME, L-arginine methyl ester; L-NA, L-N^G-nitroarginine; $AtSO_4$, atropine sulfate.
* Significantly different from vehicle control (PS + AtMN only) ($P < 0.05$).

Nitric oxide produced by activation of nitric oxide synthase following excessive stimulation of NMDA receptors was proposed as a cytotoxic molecule responsible for neuronal cell death (Dawson et al., 1991). This was supported by several studies showing that nitric oxide synthase inhibitors attenuated the seizures and neuronal damage (Dawson et al., 1991; Osonoe et al., 1994; Przegalinski et al., 1994). Thus, it seems that nitric oxide may play the role of a proconvulsant molecule in exerting seizures or neuronal damage. However, there are also reports of the anticonvulsant role of nitric oxide in quinolinate or kainate-induced seizures (Haberny et al., 1992; Rondouin et al., 1993).

Although there are still conflicting results on the role of nitric oxide, our observation, consistent with the reports in cholinergic models of epilepsy (Bagetta et al., 1992; Van Leeuwen et al., 1995), supports the role of nitric oxide as a proconvulsant or convulsion-promoting factor in DFP poisoning; L-AME potentiated the intensity of seizures induced by a high dose (2.0 LD_{50}) of DFP, which might be due to its role as a possible nitric oxide donor in DFP poisoning, as demonstrated from the effect of L-arginine in excitatory amino acid-induced seizures (De Sarro et al., 1993). An additional action of L-AME may be ascribed to the anticholinergic effect as observed by Buxton et al. (1993). Thus, it is supposed that the toxic mechanism of DFP at a high dose may involve nitric oxide-dependent system in ad-

dition to cholinergic system. A further support may come from the observations that seizures induced by a high dose of DFP was relieved by L-NAME, a nitric oxide synthase inhibitor possessing additional anticholinergic action (Buxton et al., 1993), and that L-NA, devoid of anticholinergic effect, displayed a good protection only in combination with atropine sulfate at 5 mg/kg, a dose much lower than ED_{50} in soman poisoning (Shih et al., 1991). Meanwhile, in the poisoning with a low dose (1.2 LD_{50}) of DFP, seizure potentiation was not pronounced remarkably with L-AME. This might be explained by the assumption that nitric oxide may not be important in the poisoning with a low dose of DFP. Alternatively, the inhibitory feedback action of nitric oxide on NMDA receptors may be implicated, since the convulsion intensity may be the net outcome of opposed excitatory and inhibitory processes (Herberg and Rose, 1994). The anticonvulsant effect was also obtained with procyclidine, an NMDA receptor antagonist with an anticholinergic action (Waelbroeck et al., 1990), consistent with previous reports that acetylcholine triggered DFP-induced epileptogenesis (Jones et al., 1990) and potentiated glutamate-induced neurodegeneration (Mattson, 1989). The anticonvulsant action of procyclidine may be supposed by the report (Dawson et al., 1991) that stimulation of NMDA receptors is followed by activation of nitric oxide synthase. Taken together, it is suggested that the blockade of nitric oxide production by inhibiting nitric oxide synthase or antagonizing NMDA receptors in combination with anticholinergic treatment may be required for more effective prevention of convulsions induced by anticholinesterase poisoning.

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