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Effects of sodium nitroprusside, a nitric oxide donor, on γ-aminobutyric acid concentration in the brain and on picrotoxin-induced convulsions in combination with phenobarbitone in rats

Vanaja Paul*, Perumal Ekambaram

Department of Pharmacology and Environmental Toxicology, Dr. ALM Postgraduate Institute of Basic Medical Sciences, Taramani, University of Madras, Chennai-600 113, India

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Abstract

The concentrations of nitric oxide (NO), the neuronal messenger molecule, and γ-aminobutyric acid (GABA), the inhibitory neurotransmitter, and the activity of γ-aminobutyric acid transaminase (GABA-T), the enzyme involved in the degradation of GABA, were measured in the brain of rats treated with graded doses (1.25, 2.5, 5.0 mg/kg) of sodium nitroprusside (SNP), the donor of NO. The effect of SNP was tested alone and in combination with phenobarbitone (PB), the GABA potentiating antiepileptic drug, against picrotoxin (PCT) (5 mg/kg)-induced convulsions in rats. The results of these studies showed that NO released from SNP (2.5 mg/kg) had a potential to inhibit GABA-T activity resulting in an increase in the concentration of GABA in the brain. Thus, SNP (2.5 mg/kg) was able to inhibit PCT-induced convulsions and was able to produce an additive anticonvulsant action with PB. However, a much greater increase in the concentration of NO by 5.0 mg/kg of SNP did not change the activity of GABA-T and the concentration of GABA, and promoted the convulsant action of PCT. These results suggest that a moderate increase in the concentration of NO following the administration of its donor SNP (2.5 mg/kg) results in an enhancement of the concentration of GABA in the brain and in an inhibition of PCT-induced convulsions independently and additively with PB and that a marked increase in NO concentration after the administration of a larger dose of SNP (5.0 mg/kg) results in proconvulsant action.

Keywords: Nitric oxide; γ-aminobutyric acid; Sodium nitroprusside; Picrotoxin

1. Introduction

Nitric oxide (NO), the gaseous free radical synthesized from the amino acid L-arginine by nitric oxide synthase (NOS), functions as a neuronal messenger and as a modulator of neurotransmitters in the brain (Moncada et al., 1991). NO has been proposed to function as an endogenous anticonvulsant substance since a decreased synthesis of NO in the brain following the administration of the inhibitors of NOS resulted in an exacerbation of experimentally-induced convulsions in rats (Buisson et al., 1993; Wang et al., 1994). In support of this proposal, in these studies, NO increasing doses of L-arginine prevented the inhibitors of NOS from producing proconvulsant action. Further, L-arginine inhibited sound (Smith et al., 1996) and picrotoxin (PCT) (Paul and Subramanian, 2002)-induced convulsions in rodents. Marangoz et al. (1994) have also suggested that NO has an anticonvulsant action in the brain because, in their study, intracortical microinjection of sodium nitroprusside (SNP), a donor of NO (Kowaluk et al., 1992), inhibited epileptiform discharges elicited by intracortical injection of penicillin in rats. However, formation of NO in excess has been found to produce neurotoxic action (Dawson and Dawson, 1996). In support of this finding, microinjection of L-arginine (10 nmol) deep prepiriform cortex potentiated epileptic discharge elicited by

* Corresponding author. F-1, Varalakshmi Castle, 3, Akbarabad II Street, Kodambakkam, Chennai-600 024, India.

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N-methyl-D-aspartate and kainic acid in rats (De Sarro et al., 1993). Supportingly, in a previous study in this laboratory, L-arginine, at a dose much greater than that inhibited PCT-induced convulsions, produced proconvulsant action on the same convulsion model in rats (Paul, 2002).

Systemic administration of NO increasing dose of L-arginine increased the concentration of γ-aminobutyric acid (GABA), the well established inhibitory neurotransmitter having anticonvulsant property (Silvilotti and Nistri, 1991), by inhibiting the activity of GABA metabolizing enzyme, γ-aminobutyric acid transaminase (GABA-T) in the brain (Jayakumar et al., 1999; Paul and Jayakumar, 2000). NO was found to increase the release of GABA from cerebral cortex (Kuriyama and Ohkuma, 1995), hippocampus (Lonart et al., 1995) and striatum (Segovia and Mora, 1998). Further, an increase by L-arginine of the concentration of NO and GABA in the brain coincided with an inhibition of PCT-induced convulsions in rats (Paul and Subramanian, 2002). These results provide evidence that NO synthesized from L-arginine inhibits experimentally induced convulsions by increasing the concentration and release of GABA. As a result of such a functional interaction between NO and GABA, the anticonvulsant effect of L-arginine was additive with that of phenobarbitone (PB) (Paul, 2003), the well documented GABA potentiating antiepileptic drug (Haefely, 1980). However, the anticonvulsant effect of SNP has not been correlated with the changes produced by it on the concentration of GABA and the activity of GABA-T in the brain. The concurrent anticonvulsant action of SNP and PB has also not been studied. These information are required to determine whether NO formed without the involvement of its precursor and synthesizing enzyme, inhibits convulsions by increasing GABA concentration in the brain and whether the anticonvulsant effect is additive with that of PB. In order to investigate this, the present study was designed to correlate the time- and dose-dependent effects of NO released from SNP on the concentration of GABA and the activity of GABA-T in the brain of rats. Further, the dose-dependent effect of SNP was tested independently and in combination with an anticonvulsant dose of PB against PCT-induced convulsions in rats.

2. Materials and methods

2.1. Animals

Colony bred adult (4–5 months old) male Wistar rats (130–150 g) were used. Since female rats were highly susceptible to the convulsant action of PCT due to estrous cycle (Paul and Krishnamoorthy, 1988), male rats were chosen for this study. Test (n=10) and control (n=10) groups were selected randomly. The animals were housed in groups (3 or 4 in a cage), maintained on a 12/12 h light–dark cycle at room temperature (22–26 °C) and were fed a balanced diet (Gold mohur, Mumbai, India) and tap water ad libitum. Fresh animals were used for every test. Experiments were conducted in accordance with the guidelines for breeding and experiments on animals defined by the Ministry of Social Justice and Empowerment, Government of India and the Institutional Ethical Guidelines.

2.2. Drugs and doses

The dose (5 mg/kg) of PCT (Sigma, St. Louis, MO, USA) that produced clonic convulsions and not tonus and death of animals (Paul and Krishnamoorthy, 1988) was used in this study. The graded doses (1.25, 2.5, 5.0 mg/kg) of SNP (SRL Fine Chemicals, Mumbai, India) that were tested against PCT-induced convulsions in rats, in a preliminary study in this laboratory, were chosen for this study. The minimum dose (20 mg/kg) of PB (Samarath Pharma, Mumbai, India) that inhibited PCT-induced convulsions in a previous study (Paul, 2003) was used in this study. The drugs were dissolved in physiological saline and were injected intra-peritoneally 0.2 ml/100 g body weight. The control animals received an equivalent volume of the vehicle at the same time when the test animals received SNP or PB.

2.3. Effect of SNP on the concentrations of NO, GABA and the activity of GABA-T

Five or thirty minutes after the administration of SNP, the animals were sacrificed by decapitation, whole brain was removed and processed immediately for the biochemical study. In order to measure NO concentration, nitrite (μmol/g), the stable oxidation product of NO in the brain (Moncada et al., 1991), was determined using a previously described method (Saville, 1958). This method was used in a previous study, in this laboratory, to measure NO concentration in the brain of L-arginine and L-nitro-L-arginine methyl ester (L-NAME)-treated rats (Paul and Jayakumar, 2000). Briefly, the method was based on the conversion of nitrite to *S*-nitrosothiol upon adding glutathione and formation of a brilliant azo dye after adding a reagent consisting of mercuric chloride and sulphanilamide and then *N*-1-naphthylethylene diamine. The intensity of the color was measured in a spectrophotometer at 530 nm.

GABA was measured using a previously described method (Carmona et al., 1980). This method was used previously by the authors to determine GABA concentration in the brain of L-arginine and L-NAME-treated rats (Paul and Jayakumar, 2000). As described in the method, the animals were injected intra-peritoneally with neutralized 3-mercapto-proprionic acid (100 mg/kg) 2.5 min before sacrifice in order to prevent post-mortal increase in GABA concentration.

The activity of GABA-T was determined as described previously by Hansson and Sellstrom (1983). This method was used by the authors, in a previous study, to determine GABA-T activity in the brain of L-arginine and L-NAME-treated rats (Paul and Jayakumar, 2000). Different groups were used for NO, GABA and GABA-T determinations.

2.4. Effect of SNP on PCT-induced convulsions

Five or thirty minutes after SNP treatment, the animals were injected with PCT. The time between the injection of PCT and the appearance of the first clonic convulsion movement (sudden twitching movement of the head or limbs or both) was determined for the time of onset of the convulsant action of PCT. The frequency of convulsion movements was measured in these animals, as done in a previous study in this laboratory (Paul, 2003), using the convulsion monitoring apparatus (Paul and Kazi, 1994). The capacitance sensors mounted in the floor of this instrument picked up the vibrations caused by the clonic convulsion movements of the animal and converted into electric signals which activated the counter. Soon after PCT treatment, the animal was placed in the chamber and the instrument was switched on when clonic convulsions appeared. Recording was continued as long as convulsions persisted (50–60 min aster the induction).

2.5. Effect of SNP post-treatment on the anticonvulsant action of PB

Since NO is known to produce vasodilatation (Moncada et al., 1991), a pharmacokinetic interaction is likely to occur between SNP and PB. In order to evaluate this phenomenon, in this study, the inhibitory effect of PB (20 mg/kg) on spontaneous motor activity was measured 30 min after its administration in SNP (2.5 mg/kg) pre- (5 min) and post-treated (25 min) animals. Motor activity was measured for a duration of 10 min using a conventional photoactometer. The digital counter in the instrument indicated the number of interruptions caused by the locomotor movements of the animal. SNP did not alter the motor activity countings (control, 448±32; SNP, 432±28) But, pre- and not post-treatment of it significantly enhanced the effect of PB (PB, 312±12; SNP+PB, 216±11; PB+SNP, 308±14) indicating that penetration of PB was facilitated by SNP pre- and not post-treatment. Therefore, in order to eliminate a pharmacokinetic interaction between SNP and PB, in this study, 25 min after the administration of PB, the animals were treated with SNP (2.5 mg/kg) and challenged 5 and 30 min later with PCT.

The experiments were carried out between 10:00 and 12:00 h. Convulsion tests were done at room temperature (22–26 °C). Biochemical determinations were done in a cold room (4 °C). Different groups were used for convulsion and biochemical studies. The data were compared with the respective control group and analyzed by using two-way ANOVA and Tukey's multiple comparison test. P values less than 0.05 were considered significant.

3. Results

3.1. Effects of SNP on NO, GABA and GABA-T

No significant changes were observed in the concentrations of NO and GABA and the activity of GABA-T, 5

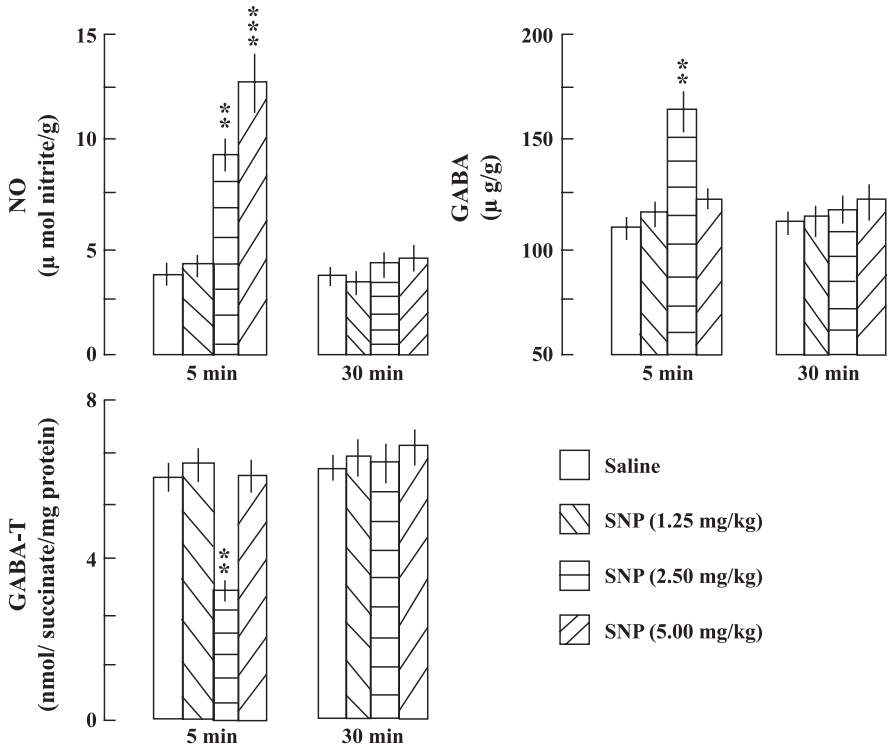

Fig. 1. Effects of SNP on the concentrations of NO and GABA and the activity of GABA-T in the brain. The data are mean±SEM of 10 animals. **P<0.01; ***P<0.001 as compared to saline treated control.

and 30 min after the administration of 1.25 mg/kg of SNP (Table 1). A significant increase in the concentrations of NO (F=5.39, P<0.01) was found 5 and not 30 min after the administration of 2.5 mg/kg of SNP. NO increasing action of 2.5 mg/kg of SNP coincided with an elevation of GABA concentration (F=6.57, P<0.01) and a decrease in the activity of GABA-T (F=5.48, P<0.01) in the brain. NO concentration was increased 5 min (F=6.58, P<0.01) and not 30 min after the administration of 5 mg/kg of SNP. GABA concentration and the activity of GABA-T were not altered significantly in the brain of these animals (Fig. 1).

3.2. Effect of SNP on PCT-induced convulsions

Clonic convulsion movements appeared 10.5±1.4 and 11.2±1.2 min after the administration of PCT in animals treated 5 and 30 min previously with saline, respectively. No clonic convulsions were observed in these animals 50–60 min after the onset of action indicating that the animals recovered from the convulsant action of PCT. Five and thirty minutes pretreatment with 1.25 mg/kg of SNP did not alter the convulsant action of PCT. A prolongation (F=5.84, P<0.01) of the time of onset of PCT-induced convulsions and a decrease (F=6.48, P<0.01) in the frequency of

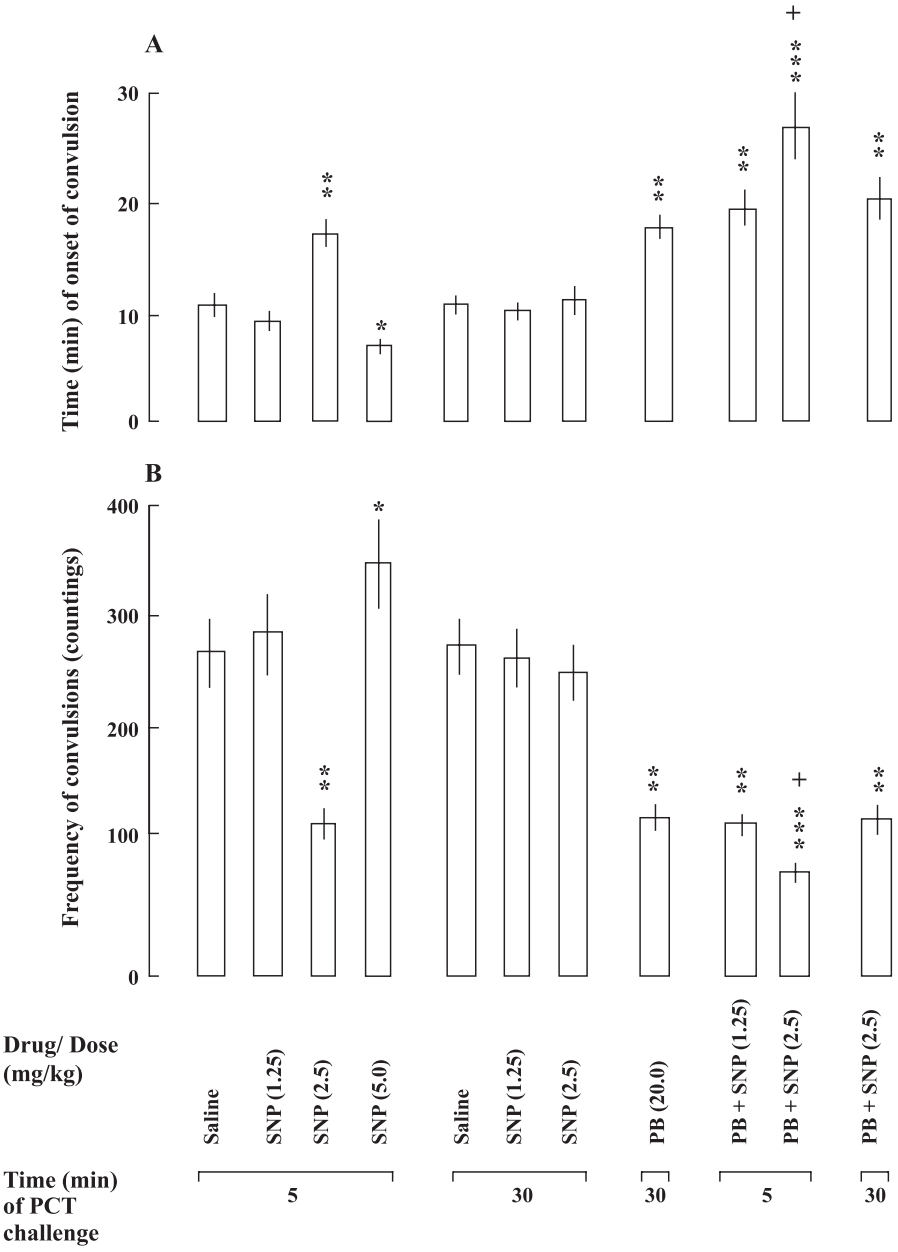

Fig. 2. The time (min) of onset of convulsions (A) and the frequency of convulsion movements (B) after the administration of PCT in animals pretreated (5 or 30 min) with saline, SNP, PB and PB+SNP. For the concurrent action of PB and SNP, SNP was administered 25 min after PB. The data are mean ±SEM of 10 animals. **P<0.01, ***P<0.001 as compared to saline treated control. ^{+}P<0.05 as compared to the independent effect of SNP or PB.

convulsions were observed in animals treated 5 min previously with 2.5 mg/kg of SNP. Thirty minutes pretreatment with the same dose of SNP produced no significant change in the convulsant action of PCT. Five min pretreatment with 5.0 mg/kg of SNP shortened the time of onset of convulsions (F=3.29, P<0.05) and increased the frequency of convulsion movements (F=3.28, P<0.05) (Fig. 2).

3.3. Effect of SNP post-treatment on the anticonvulsant action of PB

PB delayed (F=6.23, P<0.01) the onset of PCT-induced convulsions. The frequency of clonic convulsion movements was decreased in these animals (F=5.57, P<0.01). These effects of PB were not altered by post-treatment of the smaller dose (1.25 mg/kg) of SNP. However, administration of 2.5 mg/kg of SNP 5 and not 30 min before PCT challenge in PB-treated animals resulted in a greater prolongation (F=3.29, P<0.05) of the time of onset of the convulsant action of PCT and a marked decrease (F=3.32, P<0.05) in the frequency of convulsion movements than that produced by these compounds independently (Fig. 2). These results indicate that the protective effect of 2.5 mg/kg of SNP and that of PB are additive with each other.

4. Discussion

The data presented here show clearly that NO released 5 min after the administration of the larger doses (2.5 and 5.0 mg/kg) and not the smaller (1.25 mg/kg) dose of SNP increased NO concentration in the brain. However, the same doses of SNP did not increase NO concentration 30 min after their administration. Consistently, L-arginine also failed to increase the concentration of NO in a time (30–60 min)-dependent manner in the brain (Paul, 2002). It is apparent from these results that NO that is derived from its donor as well as precursor does not exist in the brain for a long time. In support of this proposal, NO was found to be metabolized quickly in the brain to nitrite, nitrate and peroxynitrite (Moncada et al., 1991).

In the present study, a moderate increase in the concentration of NO by 2.5 mg/kg of SNP resulted in an enhancement of GABA concentration in the brain and in an inhibition of the convulsant action of PCT. PCT has been reported to produce convulsions by blocking GABA-A receptor activity in the brain (Silvilotti and Nistri, 1991; Owens and Kriegstein, 2002). Surprisingly, the investigators who measured the activity of the neuronal nitric oxide synthase (nNOS) and not NO concentration in the brain of rats treated with a convulsant dose of PCT, speculated that an elevation of NO concentration by an abnormal activation of nNOS by PCT resulted in an induction of convulsions (Rajasekaran et al., 2003). If, in accordance with this speculation, NO acts as a convulsant in the brain, then NO increasing compounds are likely to produce convulsions and to potentiate the convulsant action of PCT. On the contrary, NO increase by SNP and L-arginine has resulted in an inhibition of PCT-induced convulsions in the present and in previous studies (Paul and Subramanian, 2002; Paul, 2003), respectively. These results and a decrease by the convulsant dose of PCT of the concentration of NO in correlation with an inhibition of NOS activity in the brain (Jayakumar et al., 1999; Paul et al., 2001) have been taken together to suggest that PCT induces convulsions by inhibiting NO synthesis also and that the convulsant action of PCT is preventable if NO concentration is increased in the brain. SNP and L-arginine were effective against other convulsion models too. NO increase by intracortical micro-injection of SNP inhibited epileptiform discharges elicited by penicillin in rats (Marangoz et al., 1994). L-Arginine protected genetically epilepsy prone rats and DBA/2 mice from sound-induced convulsions (Smith et al., 1996) and prevented the inhibitors of NOS from producing proconvulsant action (Buisson et al., 1993; Wang et al., 1994; Paul, 2003; Paul and Ekambaram, 2004). These results suggest that NO acts as an endogenous anticonvulsant substance in the brain. However, excess accumulation of NO was found to produce neuroexcitatory action (Dawson and Dawson, 1996). In support of this result, direct injection of NO (330–800 μmol) into the brain resulted in brief clonic convulsive episodes in rats (Smith et al., 1991). Thus, a neurotoxic action of NO may account for the proconvulsant action that resulted following a marked increase in NO concentration after the administration of the larger dose (5 mg/kg) of SNP in the present study and that produced, in a previous study, by intracortical injection of L-arginine (10 nmol) on excitatory amino acids-induced seizures in rats (De Sarro et al., 1993). Further, doses, much greater than those inhibited PCT-induced convulsion, potentiated the convulsant action of PCT in rats (Paul, 2002). Interestingly, in this study, the proconvulsant action of L-arginine was not accompanied by an excess accumulation of NO in the brain, suggesting that NO has no direct neurotoxic action. This suggestion is supported by the report that an increased accumulation of peroxynitrite, the metabolite of NO, is responsible for the neurotoxic action of NO that is generated in excess in the brain (Dawson and Dawson, 1996). An excess production by NO of cyclic guanosine monophosphate (cGMP), the synthesis of which is known to be promoted by NO (Moncada et al., 1991), may also account for the proconvulsant action of excessively formed NO because, a relationship has been found between an accumulation of cGMP and induction of convulsions in rats (Ferrendelli et al., 1980). It is apparent from these results that a moderate increase in NO concentration in the brain results in an inhibition of convulsions and that NO at a much higher concentration produces proconvulsant action.

In the present study, NO increase by the anticonvulsant dose of SNP (2.5 mg/kg) coincided with an elevation of GABA concentration and an inhibition of GABA-T in the brain. An increase in the concentration of GABA by the

anticonvulsant dose of L-arginine was also accompanied by an inhibition of GABA-T activity in the brain (Jayakumar et al., 1999; Paul and Jayakumar, 2000). In these studies, GABA increase by L-arginine did not coincide with changes in both the concentration of glutamine, the immediate precursor of GABA and the activity of glutamic acid decarboxylase, the GABA synthesizing enzyme. These results suggest that NO has a GABA increasing property by inhibiting GABA-T activity in the brain. In support of this suggestion, a decreased synthesis of NO by the inhibitor of NOS, L-NAME, was found to coincide with an increase in the activity of GABA-T and a reduction in the concentration of GABA in the brain (Paul and Jayakumar, 2000). However, production of NO in excess does not seem to increase GABA concentration, because in the present study 5 mg/kg of SNP failed to alter both GABA concentration and GABA-T activity in the brain. The neurotoxic action of excessively accumulated NO (Dawson and Dawson, 1996) has been speculated for this result.

An increase by NO of the concentration of GABA seems to be followed by an induction of the release of the neurotransmitter, because an elevation of NO concentration in the brain has resulted in an increased release of GABA from the cerebral cortex (Kuriyama and Ohkuma, 1995), hippocampus (Lonart et al., 1992) and striatum (Segovia and Mora, 1998). Conversely, L-NAME decreased the release of GABA in brain regions (Montague et al., 1994). These results and the data showing a co-localization of NO and GABA in the brain regions (Wang et al., 1997) provide strong support to the suggestion that the action of GABA which is a well established inhibitory neurotransmitter producing anticonvulsant action (Silvilotti and Nistri, 1991; Owens and Kriegstein, 2002), is activated by NO in the brain. Thus, it is likely that NO that is released from SNP inhibits PCT-induced convulsions by increasing GABA activity in the brain. In support of this suggestion, an interaction between NO and GABA was suggested, in an earlier study, for the anticonvulsant action of NO precursor, L-arginine (Paul and Subramanian, 2002). In this study, an elevation of NO concentration in the brain by L-arginine was accompanied by an increase in GABA concentration and an inhibition of PCT-induced convulsions in rats.

PB has been shown to inhibit experimentally induced convulsions by potentiating GABA activity in the brain (Haefely, 1980). Recently, Rajasekaran et al. (2003) tested the effect of PB on PCT-induced convulsions in rats pretreated with nNOS inhibitor, 7-nitroindazole (7-NI, 25 and 50 mg/kg)), in order to investigate the role of nNOS in the anticonvulsant action of these compounds. In this study, the effect of doses larger than 50 mg/kg of 7-NI was not tested either alone or in combination with PB. Further, the independent and concurrent anticonvulsant effect of 7-NI and PB was not correlated with the changes produced by these compounds on the concentration of NO, the product of nNOS activity. Without these valuable results, these investigators have speculated that a decreased nNOS activity is responsible for the anticonvulsant effect of these compounds because, in their study, the protective effect of PB on PCT-induced convulsions was potentiated by 7-NI.

7-NI (Paul and Ekambaram, 2003, 2004) and the non-selective NOS inhibitors (Rundfeldt et al., 1995) were reported to produce anticonvulsant and pro-convulsant actions on the same convulsion model in dose-dependent manner. The smaller doses of these compounds failed to decrease NOS activity and NO concentration in the brain, but inhibited experimentally induced convulsions (Rundfeldt et al., 1995; Paul, 2003; Paul and Ekambaram, 2003, 2004) and produced a greater protection concurrently with PB (Paul, 2003; Paul and Ekambaram, 2003) suggesting that the inhibitors of NOS produce anticonvulsant action by a nonspecific mechanism and that this action is additive with that of PB.

An inhibition of NO synthesis has been proposed for the anticonvulsant action of the inhibitors of NOS on pentylenetetrazol (Osonoe et al., 1994; Kaputlu and Uzbay, 1997), strychnine (Kaputlu and Uzbay, 1997) and sound (Smith et al., 1996)-induced convulsions in rodents. However, De Sarro et al. (2000) have reported that an inhibition of NO synthesis is unlikely to be responsible for the anticonvulsant action of NOS inhibitors because, in their study, the protective effect of 7-NI was not prevented by L-arginine. This result also suggests that a mechanism independent of NOS inhibition is involved in the anticonvulsant action of the inhibitors of NOS. However, a decrease by these compound of NOS activity and NO concentration in the brain has been found to produce proconvulsant action on chemically (Rondouin et al., 1993; Paul, 2003; Paul and Ekambaram, 2003, 2004) and cortical stimulation (Rundfeldt et al., 1995)-induced convulsions in rats. The proconvulsant action of L-NAME (Paul, 2003), L-nitroarginine (Rondouin et al., 1993) and 7-NI (Paul and Ekambaram, 2003, 2004) was prevented by NO increasing dose of L-arginine in rats. Further, pretreatment with NO decreasing doses of L-NAME (Borowicz et al., 1998; Paul, 2003) and 7-NI (Borowicz et al., 1997; Paul and Ekambaram, 2003) impaired the anticonvulsant effect of PB in rodents. Together, these observations suggest that an inhibition of normally occurring synthesis of NO by either nonspecific or nNOS inhibitors results in proconvulsant action and a prevention of the anticonvulsant effect of PB. In this context, the speculation of Rajasekaran et al. (2003) that 7-NI and PB inhibited PCT-induced convulsions by decreasing nNOS activity in the brain does not seem to be appropriate. Further, this speculation contradicted a previous report of Thompson et al. (1997) that nNOS activity was not altered by a single anticonvulsant dose of PB and that administration of the same dose of PB daily for 3 days resulted in an increase in both the expression of nNOS and the formation of citrulline in the brain suggesting that nNOS is inducible by PB. An induction by PB of nNOS is possible because nNOS is known to share some structural and functional similarities with the cytochrome P-450 reductase (Bredt et al., 1991;

White and Marletta, 1992) and because PB is an inducer of cytochrome P-450 enzymes (He and Fulco, 1991).

The data presented here show clearly that the protective effect of PB has been enhanced by the anticonvulsant dose of SNP. A pharmacokinetic mechanism, as a result of the vasodilator action of NO (Moncada et al., 1991), was unlikely to have a role in the greater protective effect of PB in SNP (2.5 mg/kg) post-treated (25 min) animals, because in the present study, a similar treatment of SNP did not alter the inhibitory action of PB on spontaneous motor activity in rats. The results presented here clearly show that 5 and not 30 min treatment of SNP increased GABA concentration in the brain and that SNP inhibited PCT-induced convulsions independently and additively with PB when it was administered 5 min and not 30 min before PCT challenge. These results suggest that an interaction between the GABA increasing action of SNP that has been demonstrated in the present study, and the GABA potentiating property of PB (Haefely, 1980) seems to be responsible for the additive anticonvulsant action of PB and SNP on PCT-induced convulsions. The same mechanism has been proposed earlier for the additive protective effect of PB and L-arginine against PCT-induced convulsions in rats (Paul, 2003).

In summary, the investigations carried out in the present study provide evidence that a moderate increase in the concentration of NO following a release of it by the donor, SNP results in an elevation of GABA level in the brain and in an inhibition of PCT-induced convulsions independently and additively with PB. On the other hand, a marked increase in the concentration of NO after the administration of a larger dose of SNP results in proconvulsant action.

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