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Enhancement of 7-nitro indazole-induced inhibition of brain nitric oxide synthase by norharmane

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Abstract

7-Nitro indazole (7-NI) has been used as a selective inhibitor of neuronal nitric oxide synthase (NOS) in vivo. This agent has a short duration of action which may be due to its metabolism. The structure of 7-NI resembles that of tryptophan which can be metabolized by the enzyme indolamine 2,3-dioxygenase (IDO). If 7-NI is also metabolized by this enzyme, then inhibition of IDO should augment the action of 7-NI on brain NOS activity. This possibility was examined by investigating the potential of norharmane, an IDO inhibitor, on the inhibitory effect of 7-NI on NOS catalytic activity (3, 4.5 and 7.5 h post-injection of 7-NI) in five brain regions. Norharmane, which alone did not alter NOS activity, enhanced the action of 7-NI on NOS activity in the cortex (4.5 and 7.5 h), hippocampus (3 h) and substantia nigra (3, 4.5 and 7.5 h) but not in the cerebellum or striatum. This suggests that IDO activity may, at least in part, be responsible for the relatively short duration of 7-NI action.

Keywords: 7-Nitro indazole; Indolamine 2,3-dioxygenase; Nitric oxide; Nitric oxide synthase

Nitric oxide (NO) formation through activation of nitric oxide synthase (NOS) has been implicated in many neuronal processes such as regulation of local cerebral blood flow [10], long-term potentiation [2], nociception [14] and NMDA receptor-mediated excitotoxicity [6]. To date, three isoforms of NOS have been identified which either contain or require the same cofactors [9,15]. Endothelial and neuronal NOS are constitutive enzymes which are activated by Ca^{2+}/calmodulin whereas macrophage NOS is a cytokine or endotoxin inducible enzyme [9].

In view of the potential role of NO in various neurological processes, inhibitors of NOS have been used as experimental tools to examine the effect of NO in these systems. Recently, there has been interest in agents that have the ability to selectively inhibit neuronal NOS in vivo [16]. Among such compounds, 7-nitro indazole (7-NI) and 3-bromo-7-NI have been identified as the most potent inhibitors of neuronal NOS activity by binding to the prosthetic heme moiety in a competitive manner with the substrate L-arginine [13,21]. Although, 7-NI inhibits all NOS isozymes in vitro [1,21], when administered to animals, it appears to selectivity inhibit neuronal NOS [1,16]. However, the duration of action of 7-NI is shorter [12] than that of non-specific arginine-based inhibitors such as N^{ω}-nitro-L-arginine methyl ester [11].

The short duration of 7-NI action may be due to its rapid metabolism. The structure of 7-NI has a resemblance to that of the amino acid tryptophan (Fig. 1). In the CNS, the phenyl ring of tryptophan can be hydroxylated by tryptophan hydroxylase [5] or the indole ring can be cleaved by indolamine 2,3-dioxygenase (IDO) to form formylkynurenine, a reaction which is part of the kynurenine biosynthetic pathway [19]. The prominence of the kynurenine pathway in tryptophan metabolism is reflected in the finding that more than 95% of dietary tryptophan is converted to kynurenines as opposed to serotonin [20]. If 7-NI is metabolized by IDO, then inhibition of this enzyme can be expected to enhance or prolong its inhibitory action on NOS activity. To test this hypothesis, we examined the ability of an IDO inhibitor, norharmane, to influence the action of 7-NI on NOS catalytic activity in five different brain regions.

7-NI (Research Biochemicals Inc., Natick, MA) and norharmane (Sigma, St. Louis, MO) were both prepared

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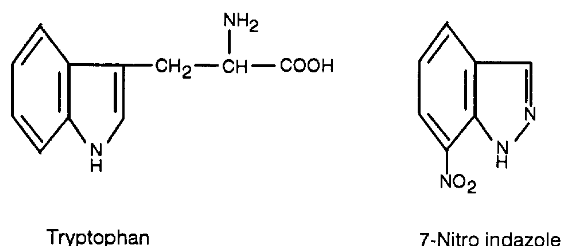

Fig. 1. Structures of tryptophan and 7-nitro indazole.

by sonication in sesame oil. Male Sprague–Dawley rats (250–275 g) were given a single injection of vehicle (sesame oil, i.p.) or 7-NI (20 mg/kg, i.p.) and sacrificed at 3, 4.5 or 7.5 h post-injection. Animals pretreated with norharmane were given a single injection of norharmane (100 mg/kg, i.p.) 30 min prior to injection of vehicle or 7-NI. After sacrifice, the brain was removed and placed in ice-cold saline. The cerebellum, striatum, cortex, hippocampus and substantia nigra were removed and homogenized with a Teflon pestle in a solution containing 1 mM DTT, 10 μg/ml leupeptin, 1 mM EDTA and 50 mM HEPES buffer at pH 7.4. The homogenates were centrifuged at 20 000 × g for 30 min at 4°C and the supernatant retained for the NOS assay.

NOS activity was assayed using a modification of the method described by Bredt and Snyder [4] which measures the amount of [^{14}C]arginine converted to [^{14}C]citrulline. Tissue samples (25 μl) were incubated for 1 h at 37°C with 25 μl of 100 μM [^{14}C]arginine (0.319 Ci/mmol, 50 μCi/ml; Amersham, Oakville, Ontario) and 100 μl of reaction buffer. The reaction buffer consisted of 1 mM DTT, 1.25 mM $CaCl_2$, 1 mM valine, 2 mM NADPH, 1 mM EDTA and 50 mM HEPES buffer (pH 7.4). The reaction was terminated by the addition of 2 ml of ice-cold stop buffer which contained 2 mM EDTA and 20 mM HEPES buffer at pH 5.5. Each sample was passed through a 1 ml 50W-X8 cation exchange column (Na^+ form) which was washed with 2 ml of distilled water and the eluate collected and counted using a Beckman scintillation counter. Protein was determined by the method of Bradford [3] and the enzyme activity was expressed as nmol citrulline formed/mg protein per h.

Basal NOS activity in vehicle treated animals was found to be 11.1 ± 0.6, 2.9 ± 0.5, 2.8 ± 0.6, 1.5 ± 0.3 and 3.7 ± 0.5 nmol citrulline/mg protein per h in the cerebellum, striatum, cortex, hippocampus and substantia nigra, respectively. The % basal NOS activity in each of these regions following injection of norharmane alone (3.5 h prior to sacrifice) was found to be 88.1 ± 2.8, 90.0 ± 12.4, 80.95 ± 15.8, 98.5 ± 10.4 and 101.2 ± 1.9 in the cerebellum, striatum, cortex, hippocampus and substantia nigra, respectively. None of these values were significantly different from corresponding values in vehicle treated animals.

The effect of 7-NI (20 mg/kg) on regional brain NOS activity in the presence and absence of norharmane (100 mg/kg) pretreatment is represented in Fig. 2. At this dose of 7-NI, only cerebellar NOS activity was significantly decreased at all three time points (Fig. 2A). Pretreatment with norharmane did not alter the effect of 7-NI on either cerebellar or striatal NOS catalytic activity (Fig. 2A,B) even though cerebellar but not striatal NOS activity was decreased at these time points. However, norharmane treatment significantly enhanced the 7-NI-induced decrease in NOS activity in the cortex (4.5 and 7.5 h), hippocampus (3 h) and substantia nigra (3, 4.5 and 7.5 h) (Fig. 2C–E).

In the present study, it was demonstrated for the first time that inhibition of brain NOS by 7-NI can be enhanced in certain brain regions following pretreatment with the IDO inhibitor, norharmane.

In addition to metabolizing tryptophan to formylkynurenine, IDO also metabolizes L-hydroxytryptophan, tryptamine, serotonin and melatonin, all of which have indole ring structures [19]. This enzyme is distributed in the intestine, lung and is widely distributed throughout the CNS [19]. Since IDO has broad substrate specificity and 7-NI has a similar ring structure to tryptophan, we hypothesized that IDO may also be metabolizing 7-NI.

Norharmane, an endogenous β-carboline, has been reported to be an non-competitive inhibitor of rabbit intestinal IDO (K_i = 0.12 mM) [8] as well as inhibiting IDO activity in macrophage cultures (IC_{50} = 43 μM) [17]. It is thought that various β-carbolines, such as norharmane, may be formed in vivo through carbonyl condensation/oxidations of tryptamine or its derivatives [7]. Systemic administration of norharmane to rats produced symptoms such as sedation, limb paresis and whisker twitching, suggesting that injected norharmane reached the CNS. Administration of norharmane to vehicle treated animals did not result in an inhibition of NOS activity in any brain region and therefore, at the dose used, does not appear to be an inhibitor of NOS activity.

MacKenzie et al. [12] reported that intraperitoneal administration of 30 mg/kg 7-NI resulted in maximal inhibition of NOS activity in five brain regions and that the activity started to return towards basal levels at 6 h post-injection. Therefore, to investigate the potential contribution of IDO in the metabolism of 7-NI, in the present study, a submaximal dose of 7-NI (20 mg/kg) was examined at 3, 4.5 and 7.5 h post-injection. Using this treatment regimen, cortical, hippocampal and nigral NOS activity showed an increase in 7-NI-induced inhibition of NOS activity in the presence of norharmane, whereas activity of cerebellar and striatal NOS was not altered by norharmane treatment. The differential effect of norharmane on 7-NI action in the various brain regions examined may be due to factors such as: (1) decreased access of norharmane into the cerebellum and striatum relative to the other brain regions; (2) norharmane itself may be

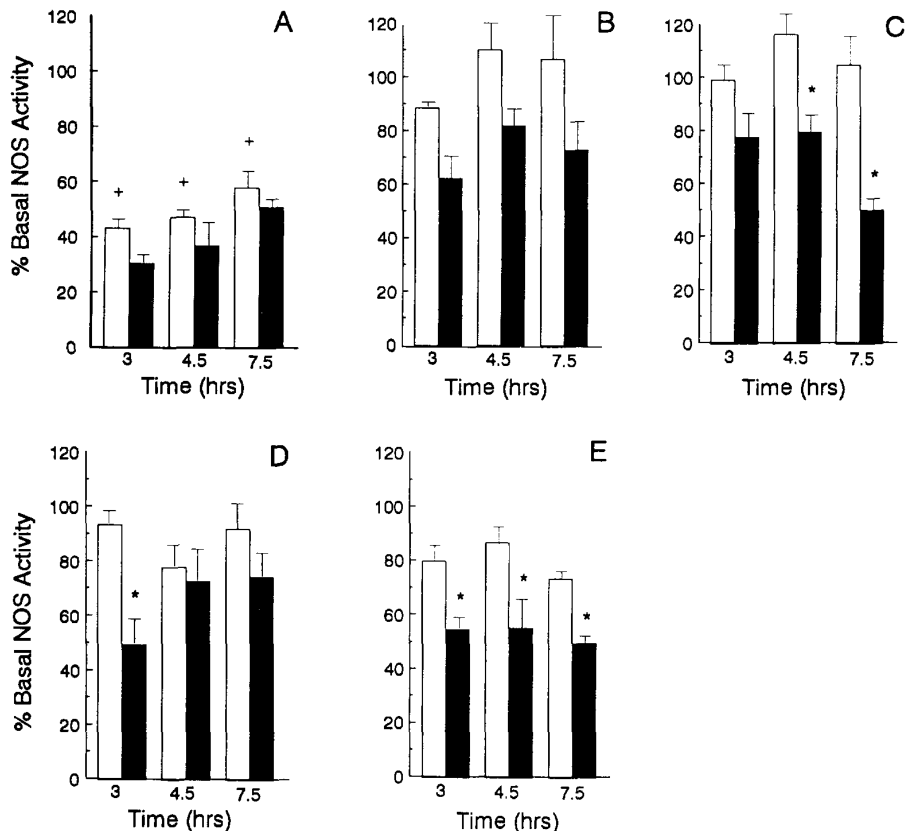

Fig. 2. Effect of norharmane pretreatment on 7-NI-induced inhibition of brain NOS. Values are expressed as percent basal NOS catalytic activity measured 3, 4.5 and 7.5 h post-injection of 7-NI (20 mg/kg, i.p.) in the cerebellum (A), striatum (B), cortex (C), hippocampus (D) and substantia nigra (E) in the presence of vehicle (□) and norharmane (100 mg/kg, i.p.) (■) pretreatment. Data are expressed as mean ± SEM with $n \geq 3$. + indicates a significant difference between vehicle and 7-NI treated animals. * indicates a significant difference between 7-NI treated animals in the presence of vehicle or norharmane pretreatment. Statistics were performed by a one-way ANOVA with Newman–Keuls post-hoc test ($P < 0.05$).

metabolized differentially in these regions; or (3) lower levels of IDO in the cerebellum and striatum compared with areas where the effect of 7-NI was enhanced – cortex, hippocampus and substantia nigra. However, the latter may not be the case since Saito et al. [18] reported that basal IDO activity in the gerbil was similar in the cerebellum, striatum, cortex, hippocampus and thalamus (approximately 1.9 nmol/g per h).

In conclusion, it appears that IDO activity may, in part, contribute to the relatively short duration of action of 7-NI in vivo although this needs to be demonstrated in future experiments. Since, 7-NI is selective for neuronal NOS in vivo and serves as a tool to examine the role of NO in CNS function, the inhibition of IDO may be useful in enhancing its action in certain brain regions.

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