Inhibition of rat cerebellar nitric oxide synthase by 7-nitro indazole and related substituted indazoles

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1 7-Nitro indazole (7-NI) produces potent inhibition of rat cerebellar nitric oxide synthase (NOS) with an IC_{50} of $0.9 \pm 0.1 \mu M$ ($n = 6$). NOS activity is dependent on the presence of both exogenous $CaCl_2$ and NADPH. The inhibitory potency of 7-NI remained unaltered in the presence of different concentrations of either $CaCl_2$ (0.75–7.5 mM) or NADPH (0.05–5.0 mM).

2 Kinetic (Lineweaver-Burke) analysis of the effect of 7-NI on rat cerebellar NOS revealed that inhibition was of a competitive nature with a K_i value of $5.6 \mu M$. The K_m of cerebellar NOS with respect to L-arginine was $2.5 \mu M$.

3 The following indazole derivatives (IC_{50} values shown in parentheses, all $n = 6$) caused concentration-related inhibition of rat cerebellar NOS *in vitro*: 6-nitro indazole ($31.6 \pm 3.4 \mu M$), 5-nitro indazole ($47.3 \pm 2.3 \mu M$), 3-chloro indazole ($100.0 \pm 5.5 \mu M$), 3-chloro 5-nitro indazole ($158.4 \pm 2.1 \mu M$) and indazole ($177.8 \pm 2.1 \mu M$). The IC_{50} values for 5-amino indazole, 6-amino indazole and 6-sulphanilimido indazole were in excess of 1 mM; 3-indazolinone was inactive.

4 7-NI (10 mg kg^{-1}) administered i.p. to rats produced 60 min thereafter a significant inhibition of NOS activity in cerebellum ($31.1 \pm 3.2\%$, $n = 6$), cerebral cortex ($38.2 \pm 5.6\%$, $n = 6$), hippocampus ($37.0 \pm 2.8\%$, $n = 6$) and adrenal gland ($23.7 \pm 3.0\%$, $n = 6$). NOS activity in olfactory bulb and stomach fundus were unchanged.

5 These results indicate that 7-NI is a potent and competitive inhibitor of rat brain NOS *in vitro* and also inhibits NOS in different brain regions and in the adrenal gland *in vivo*. Inhibition of NOS is a characteristic property of the indazole nucleus. Nitration of the indazole ring at positions 5, 6 and 7 results in a graded increase in inhibitory potency. Indazole-based inhibitors of NOS may prove useful tools with which to evaluate the biological roles of nitric oxide in the central nervous system.

Keywords: 7-Nitro indazole; substituted indazole derivatives; cerebellar nitric oxide synthase; nitric oxide; rat cerebellum; rat spleen; bovine endothelial cells

Introduction

We have recently shown that 7-nitro indazole (7-NI) inhibits rat and mouse cerebellar nitric oxide synthase (NOS) with equivalent potency to established inhibitors of this enzyme such as L-N^G-nitro arginine methyl ester (L-NAME) and L-N^G-monomethyl arginine (L-NMMA) (Moore *et al.*, 1993a). In the accompanying paper (Moore *et al.*, 1993b) we provide evidence that 7-NI, 6-nitro indazole and indazole elicit antinociception in the mouse utilising the formalin-induced hind-paw licking and acetic acid induced-abdominal constriction assays. None of the indazole derivatives investigated influenced mouse blood pressure. In this paper we have compared the ability of indazole plus nine mono- or di-substituted derivatives of indazole to inhibit rat brain cerebellar NOS *in vitro* and have further characterized the mechanism of action and isoform selectivity of 7-NI both *in vitro* and following i.p. administration in the rat.

Methods

Preparation and assay of rat cerebellar NOS

Male rats (Sprague-Dawley, 200–300 g) were killed by a blow to the head and exsanguination. Cerebella were removed, weighed and homogenized in an Ultra-Turrax (type 18/2N) homogenizer in 10 volumes of 20 mM Tris HCl buffer (pH 7.4) containing 2 mM EDTA. In some experiments, cerebella were stored at -70°C until required. Homogenates were centrifuged (10,000 g) for 15 min at 4°C . NOS was assayed by monitoring the conversion of [^3H]-arginine to [^3H]-citrulline as described by Dwyer *et al.* (1991). Incuba-

tions (15 min at 0°C followed by 15 min at 37°C) routinely contained 25 μl cerebellar supernatant, 0.5 μCi [^3H]-L-arginine (concentration = 120 nM), 0.75 mM $CaCl_2$, 0.5 mM NADPH and 5 μl water or drug solution in a total volume of 105 μl . In some experiments the concentrations of NADPH and $CaCl_2$ were varied from 0.5–5.0 mM and 0.37–7.5 mM respectively. In order to determine the K_m of rat cerebellar NOS for L-arginine and the K_i for 7-NI against this enzyme the concentration of L-arginine in the incubation medium was also varied in some experiments from 0.1–10 μM . Preliminary investigation of the time course of NOS activity under these conditions revealed that [^3H]-citrulline formation was linear up to 5 min.

Accordingly, for these experiments incubations were carried out at 37°C for 3 min. All assays were terminated by addition of 3 ml HEPES buffer (pH 5.5) containing 2 mM EDTA and incubates applied to 0.5 ml columns of Dowex AG50WX-8 (Na^+ form) followed by 0.5 ml distilled water to remove unchanged [^3H]-arginine. [^3H]-citrulline was quantified by liquid scintillation spectroscopy of a 1 ml aliquot of the combined flow-through. Protein concentration was measured by the method of Lowry *et al.* (1951).

Preparation of rat spleen and bovine endothelial cell NOS

Male rats (Sprague-Dawley, 220–250 g) were anaesthetized by i.p. administration of urethane (10 g kg^{-1}). Spleen NOS enzyme was induced by i.p. administration of 5.0 mg kg^{-1} *E. coli* lipopolysaccharide (serotype 0127:B8) and animals killed by cervical dislocation 6 h later. The spleen was removed, weighed, homogenized in 20 mM Tris-HCl buffer (pH 7.4) and assayed for NOS as described above with the exception

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that CaCl_2 was omitted from the incubation medium. Bovine aortae were obtained from a local slaughterhouse. Vascular endothelial cells were removed by careful rubbing of the intimal surface with a scalpel blade. The crude cell suspension was washed twice with phosphate buffered saline, centrifuged (1000 g, 5 min, 4°C) and endothelial cells subsequently homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA and 0.1% 2-mercaptoethanol with 25 passes of a glass teflon Dounce homogenizer. Crude homogenate was subsequently assayed for NOS as described above.

Pretreatment of rats with 7-NI

Male rats (Sprague-Dawley, 200–220 g) were injected i.p. with 7-NI (10 mg kg⁻¹) or an appropriate volume of vehicle (arachis oil, 0.1 ml 100 g⁻¹) and killed 60 min later by a blow to the head and exsanguination. Brains were removed and the cerebellum, hippocampus, cerebral cortex and olfactory bulb dissected. Stomach fundus and adrenal medulla were also removed. All brain regions/organs were weighed and homogenized in 20 mM Tris-HCl buffer (pH 7.4). In preliminary experiments low levels of NOS were detected in cerebral cortex, stomach fundus and adrenal gland. In order to optimize [³H]-citrulline production all brain regions/organs used in this part of the study were applied to 0.3 ml columns of Dowex AG50WX-8 to remove endogenous L-arginine prior to assay for NOS as described above.

Drugs and chemicals

Indazole, 5-nitro indazole, 6-nitro indazole, 5-amino indazole, 6-amino indazole, 3-chloro indazole, 3-chloro 5-nitro indazole, 3-indazolinone and 6-sulphanilimido indazole were purchased from Aldrich Ltd. 7-Nitro indazole was obtained from MTM Lancaster Ltd. Radiolabelled [³H]-arginine (sp. act. 62 Ci mmol⁻¹) was obtained from Amersham. L-N^G-nitro arginine methyl ester hydrochloride (L-NAME), L-arginine hydrochloride and NADPH were purchased from Sigma Ltd. Dowex AG50WX-8 H⁺ form (Sigma Ltd.) was converted into the Na⁺ form by soaking for 2 h in 2 M NaOH. For *in vivo* experiments 7-NI was dissolved in arachis oil. For *in vitro* experiments 7-NI and all other indazole derivatives were dissolved in Na₂CO₃ (0.5%, w/v). Drug stocks were prepared fresh on the morning of each experiment.

Statistical analysis

Results indicate mean $\pm$ s.e.mean. Statistical significance of differences between groups was determined by unpaired Student's *t* test. A probability (*P*) value of 0.05 or less was taken to indicate statistical significance.

Results

Effect of indazole derivatives on rat cerebellar NOS

Three of the ten indazole derivatives examined in this study (5-amino indazole, 6-amino indazole and 6-sulphanilimido indazole) failed to achieve >50% inhibition of NOS activity at the highest concentration employed (1 mM). Calculated IC₁₅s for these derivatives were 237.1 $\pm$ 20.9 μM , 501.2 $\pm$ 25.4 μM and 133.3 $\pm$ 9.9 μM (all *n* = 6) respectively. A fourth compound, 3-indazolinone, failed to inhibit rat cerebellar NOS at a concentration of 1 mM. Concentration-inhibition curves for the remaining indazole derivatives examined are shown in Figure 1a, b. The rank order of potency with IC₅₀ concentrations shown in parentheses (all *n* = 6) were as follows: 7-nitro indazole (0.9 $\pm$ 0.1 μM) > 6-nitro indazole (31.6 $\pm$ 3.4 μM) > 5-nitro indazole (47.3 $\pm$ 2.3 μM) > 3-chloro indazole (100.0 $\pm$ 5.5 μM) > 3-chloro 5-nitro indazole

Figure 1 Concentration-response curves for inhibition of rat cerebellar nitric oxide synthase (NOS) activity by (a), 7-nitro indazole 7-NI (■); 6-nitro indazole (□); 5-nitro indazole (●) and (b), 3-chloro indazole (▲); 3-chloro 5-nitro indazole (△) and indazole (○). Results show % inhibition of NOS and are mean $\pm$ s.e.mean, *n* = 6.

(158.4 $\pm$ 2.1 μM) > indazole (177.8 $\pm$ 2.1 μM). For comparison, the IC₅₀s for other inhibitors of NOS under identical experimental conditions were L-NAME, 0.9 $\pm$ 0.08 μM and L-NMMA, 3.4 $\pm$ 0.4 μM (*n* = 6).

Effect of substrate and cofactor concentration on inhibition of rat cerebellar NOS by 7-NI

In an attempt to obtain further information about the mechanism of action of 7-NI, additional experiments were undertaken to examine the NOS inhibitory effect of this derivative in the presence of varying concentrations of NADPH, CaCl_2 and L-arginine.

In the absence of added NADPH, rat cerebellar NOS activity was reduced by >99% (0.04 $\pm$ 0.02 pmol citrulline mg⁻¹ 15 min⁻¹, cf. 17.3 $\pm$ 0.4 pmol citrulline mg⁻¹ 15 min⁻¹ in the presence of 0.5 mM NADPH, *n* = 6, *P* < 0.005). A lower concentration of NADPH (0.05 mM) did not significantly alter NOS activity (17.9 $\pm$ 0.6 pmol citrulline mg⁻¹ 15 min⁻¹, *n* = 6, *P* > 0.05) whilst at higher concentration (5 mM) NOS activity was reduced (9.5 $\pm$ 0.5 pmol mg⁻¹ 15 min⁻¹, *n* = 6, *P* < 0.01). An approximate IC₅₀ concentration of 7-NI (1 μM) produced similar % inhibition of NOS regardless of the concentration of NADPH in the incubation (e.g. 0.05 mM, 51.6 $\pm$ 0.5%; 0.5 mM, 53.9 $\pm$ 2.7%; 5.0 mM, 50.3 $\pm$ 2.3%, all *n* = 6). Reducing the concentration of added

CaCl₂ in the incubation to 0.37 mM or 0.17 mM completely abolished rat cerebellar NOS activity (data not shown). It should be emphasised that these values do not reflect free calcium concentration in the incubation which were not measured in the present study but would be expected to be considerably lower. At higher CaCl₂ concentrations, 7-NI (10 μ M) produced similar inhibition of rat cerebellar NOS regardless of the concentration of CaCl₂ in the incubation (e.g. 0.75 mM, $76.4 \pm 0.7\%$; 1.8 mM, $74.6 \pm 0.7\%$; 3.7 mM, $87.2 \pm 2.0\%$; 7.5 mM, $82.8 \pm 0.6\%$, all $n = 6$). Clearly, it was not possible to assess the effect of 7-NI in the presence of CaCl₂ concentrations lower than 0.75 mM.

In order to investigate the possible interaction of 7-NI with a substrate binding site on rat cerebellar NOS, experiments were conducted in the presence of 0.1–10.0 μ M L-arginine. A double reciprocal (Lineweaver-Burke) plot of cerebellar NOS in the presence and absence of 7-NI (10 μ M) is shown in Figure 2. The calculated K_m for L-arginine was 2.5 μ M whilst the K_i for 7-NI was 5.6 μ M.

Effect of 7-NI on NOS from other tissue sources

Both spleen removed from endotoxin pretreated (induced) rats and bovine endothelial cells exhibited significant NOS activity (0.98 ± 0.14 pmol citrulline mg^{-1} 15 min^{-1} and 3.7 ± 0.14 pmol citrulline mg^{-1} 15 min^{-1} , respectively, $n = 6$). 7-NI inhibited rat spleen (IC_{50} , $57.0 \pm 5.5 \mu\text{M}$, $n = 6$) and bovine endothelial cell (IC_{50} , $0.7 \pm 0.2 \mu\text{M}$, $n = 6$) NOS. 7-NI was more potent than L-NAME as an inhibitor of the endothelial cell (cf. IC_{50} for L-NAME, $6.5 \pm 1.1 \mu\text{M}$, $n = 6$) but less potent as an inhibitor of the induced spleen enzyme (cf. IC_{50} for L-NAME, $9.0 \pm 0.7 \mu\text{M}$, $n = 6$).

Inhibition of NOS following administration of 7-NI in the intact rat

NOS activity in cerebellum, cerebral cortex and hippocampus was reduced by approximately 30–38% 60 min after i.p. administration of 7-NI (10 mg kg^{-1}). In contrast, adrenal NOS activity was reduced by only $23.9 \pm 2.8\%$ ($n = 6$). Olfactory bulb and stomach fundus NOS activity were unchanged (Table 1).

Figure 2 Double reciprocal analysis of the inhibitory effect of 7-NI (10 μ M) against rat cerebellar NOS using increasing concentrations of L-arginine (0.1–10 μ M) as substrate: (●) experiments carried out in the absence and (○) in the presence of 7-NI. Results show the reciprocal of the initial rate of [³H]-citrulline formation monitored over the first 3 min incubation plotted against the reciprocal of L-arginine concentration in μ M. Data indicate the mean results from 3 separate experiments in which NOS activity was monitored in triplicate. Standard errors fall within the dimensions of the symbols.

Table 1 Effect of 7-nitro indazole (7-NI) administered i.p. in rats on nitric oxide synthase (NOS) activity in different brain regions/organs

	Citrulline biosynthesis	
	Control	7-NI
Cerebellum	40.5 ± 1.8	$27.9 \pm 1.3^*$
Cerebral cortex	8.9 ± 0.3	$5.5 \pm 0.5^*$
Hippocampus	10.8 ± 0.5	$6.8 \pm 0.3^*$
Olfactory bulb	16.4 ± 0.8	17.7 ± 0.8
Adrenal gland	13.5 ± 0.4	$10.3 \pm 0.4^*$
Stomach fundus	7.1 ± 0.4	7.6 ± 0.5

NOS activity is expressed as citrulline formation (pmol mg^{-1} protein 15 min^{-1}) in organs removed from rats 60 min following i.p. injection of 7-NI (10 mg kg^{-1}) or arachis oil (0.1 ml kg^{-1} , control). Results show mean $\pm$ s.e.mean of 6 observations.

* $P < 0.05$.

Discussion

The present data indicate that 7-NI inhibits rat cerebellar NOS with a potency similar to that of L-NAME and L-NMMA (Moore *et al.*, 1993a). The principal aim of the present investigation has been to provide a more detailed characterization of the NOS inhibitory effect of 7-NI and related indazole compounds and to investigate the effect of 7-NI on NOS activity in a range of organs *in vivo*.

Preliminary experiments demonstrated that the crude (10,000 g) rat cerebellar NOS used in this study required exogenous NADPH and Ca^{2+} for activity. Optimum enzyme activity occurred at a concentration of 0.05–0.5 mM NADPH and 0.75 mM CaCl₂. The absolute dependence of brain NOS on exogenous calcium has been noted previously (Knowles *et al.*, 1989; Mayer *et al.*, 1990) although the finding that high concentrations of NADPH inhibit cerebellar NOS does not appear to have been reported. The explanation of this phenomenon is not clear although it is conceivable that crude rat cerebellar supernatant may contain additional enzymes capable of oxidising NADPH into NADP which, in turn, may inhibit NOS. Kinetic analysis of cerebellar NOS revealed a K_m for L-arginine of 2.5 μ M which is similar to previously published data for this enzyme from rat cerebellum (Schmidt *et al.*, 1991), rat cerebral cortex (Knowles *et al.*, 1989), bovine endothelial cells (Pollock *et al.*, 1991) and mouse macrophages (Stuehr *et al.*, 1991).

Reported inhibitors of NOS generally fall into one of three main classes, (a) guanidino-monomonsubstituted substrate analogues such as L-NAME and L-NMMA, (b) calmodulin antagonists (Bredt & Snyder, 1990) and (c) agents which interfere with the binding of cofactor, NADPH, such as diphenylene iodonium and its derivatives (Stuehr *et al.*, 1991). To this list may be added agents which sequester free Ca^{2+} since, as indicated in these experiments, rat cerebellar NOS has an absolute requirement for this cation. Of these possible mechanisms of action of 7-NI, an interaction with the NADPH binding site can be excluded since varying the concentration of NADPH in the incubation medium did not influence the inhibitory potency of 7-NI. Furthermore, inhibition of NOS by 7-NI was unaltered in the presence of a range of CaCl₂ concentrations implying that sequestration of Ca^{2+} ions (i.e. an EDTA-like effect) is not involved. Although not investigated in the present study we also believe it unlikely that calmodulin antagonist activity contributes to the mechanism of action of 7-NI since the concentration of calmodulin antagonists required to inhibit NOS are considerably greater than the equi-effective concentration of 7-NI (Bredt & Snyder, 1990). Furthermore, chlorpromazine, a known calmodulin antagonist, exhibits only very weak inhibition of rat cerebellar NOS (e.g. 100 μ M, $22.6 \pm 0.8\%$ inhibition of [³H]-citrulline formation, Babbedge & Moore, unpublished).

In contrast, we present here direct evidence from double reciprocal analysis of the inhibitory effect of 7-NI in the presence of varying concentrations of L-arginine that inhibition results from a competitive interaction of 7-NI with the L-arginine binding site on cerebellar NOS. The calculated K_i for 7-NI is in the low micromolar range. It could also be argued that the relatively short-lived inhibition of cerebellar NOS following i.p. administration of 7-NI in the mouse (Moore *et al.*, 1993b) provides further evidence for a competitive (reversible) mechanism of action although, of course, we cannot exclude the possibility that this apparently transient effect of 7-NI relates not to its interaction with NOS but to rapid metabolic breakdown.

The effect of 7-NI on NOS in supernatants prepared from a variety of tissue sources has also been investigated. Compared with L-NAME, 7-NI has equivalent or greater potency as an inhibitor of the constitutive isoform of this enzyme found in rat cerebellum and bovine endothelial cells. In contrast, 7-NI is some 6 times less potent than L-NAME against the inducible isoform of the enzyme located in the spleen from endotoxin-pretreated rats. Clearly, further experiments are required to determine the ability of 7-NI to inhibit the inducible isoform of NOS in, for example, leucocytes or vascular smooth muscle cells.

Administration of 7-NI to intact rats has provided further information about the tissue-specific effect of this inhibitor on a number of different NOS enzymes within the body. Thus, 60 min after i.p. administration of 7-NI (10 mg kg⁻¹), NOS activity was reduced to an almost equal extent in rat cerebral cortex, cerebellum and hippocampus and to a lesser extent in adrenal gland. In contrast, NOS activity in olfactory bulb and stomach fundus were not significantly reduced. At first sight these data appear to provide further evidence for a differential inhibitory effect of 7-NI on NOS in different target organs. However, we cannot rule out the possibility that inhibition of NOS following 7-NI administration reflects the relative blood flow and/or uptake (and hence exposure to 7-NI) or individual brain regions as well as stomach fundus and adrenal gland. Clearly, a better understanding of the uptake and distribution of 7-NI following parenteral administration in different species is necessary. Such experiments in the mouse and rat are currently in progress in this laboratory. Whatever the precise mechanism it is clear from this study that 7-NI administration *in vivo* preferentially inhibits NOS in selected brain regions.

Preliminary evaluation of the structure-activity relationship for inhibition of rat cerebellar NOS using a series of chemically related indazole derivatives has been undertaken. Examination of the ability of ten indazole derivatives to inhibit NOS reveals that nitration of the benzene ring at the 5, 6 or 7 positions results in the production of potent inhibitors of this enzyme. Ring substitution with an -NO₂ group has an electron withdrawing effect from the benzene ring and may therefore lead to the formation of a more polarised molecule. The precise position of the -NO₂ within the ring is also clearly of importance with 7-NI some 35 times and 55 times more potent than the corresponding 6-nitro and 5-nitro derivatives respectively. Interestingly, amination of the benzene ring at position 5 or 6 effectively removes the residual NOS inhibitory effect of the parent molecule, indazole, whilst chlorination at position 3 of the pyrazole ring significantly reduces the inhibitory effect of 5-nitro indazole. Since ring substitution with either -NO₂ or -Cl groups has the opposite effect to nitration viz. an electron donating function, it is tempting to suggest that indazole derivatives substituted with alternative electron withdrawing groups such as a tertiary amine or perhaps cyanoindazole derivatives may also exhibit potent NOS inhibitory activity.

The present study provides evidence that 7-NI is a competitive inhibitor of rat cerebellar NOS with potency similar to or greater than that of L-NAME and L-NMMA. Of the indazole compounds tested, 7-NI is the most potent NOS inhibitor. This observation, coupled with potent antinociceptive activity and lack of cardiovascular and other side effects *in vivo* within this group provides additional impetus for the development of novel indazole-based NOS inhibitors with which to study the central pharmacology of the NO system. Moreover, the possibility that 7-NI, or a chemically related compound, may also prove of clinical benefit in analgesia has been discussed in the accompanying paper (Moore *et al.*, 1993b). Information provided here concerning the structure-activity relationship for indazole based NOS inhibitors may prove useful in the search for such novel therapeutic agents.

We would like to thank the MRC for financial support. R.C.B. is an SERC-CASE scholar with Wellcome Research laboratories. P.B.-W. is supported by a studentship from Glaxo Group Research.

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(Received February 10, 1993

Revised April 29, 1993

Accepted May 6, 1993)