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Relatively selective neuronal nitric oxide synthase inhibition by 7-nitroindazole in monkey isolated cerebral arteries

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Abstract

The selectivity of 7-nitroindazole in inhibiting endothelial and neuronal nitric oxide synthases (eNOS and nNOS) was investigated by comparing its inhibitory action on relaxations mediated by nitric oxide (NO) in response to stimulation of perivascular nerves and in response to histamine in monkey cerebral artery strips. 7-Nitroindazole at 2×10^{-5} M moderately attenuated the response to transmural electrical stimulation and to nicotine, but did to alter the endothelium-dependent relaxation in response to histamine in cimetidine-treated strips. Raising the concentration of 7-nitroindazole to 10^{-4} M abolished the neurogenic response, partially inhibited the histamine-induced relaxation, but did not affect the response to NO. It is concluded that 7-nitroindazole is a relatively selective nNOS inhibitor; however, at high concentrations, it inhibits eNOS in monkey cerebral arteries.

Keywords: Nitric oxide (NO); Nitric oxide synthase (NOS) inhibitor; Vasodilator nerve; Cerebral artery; (Monkey)

1. Introduction

Nitric oxide (NO) is widely recognized as an important physiological substance in controlling cardiovascular and central and peripheral neuronal functions. The constitutive NO synthase is classified into endothelial and neuronal forms based on the chemical structure and location (Förstermann et al., 1991), termed endothelial and neuronal nitric oxide synthases (eNOS and nNOS; Moncada et al., 1997), respectively. In order to distinguish the physiological role of NO synthesized by these isoforms, investigators are anxious to develop the selective NOS inhibitors. 7-Nitroindazole is the most promising nNOS inhibitor so far introduced (Moore et al., 1993a). Its selectivity is shown by the fact that 7-nitroindazole, in doses sufficient to inhibit the activity of NOS purified from the cerebellum and the function mediated by nerve-derived NO, does not induce hypertension or blunt acetylcholine-induced vasodilatation in anesthetized rats and mice, nor does it interfere with the vasorelaxant effect of acetylcholine in rabbit aortic rings (Moore et al., 1993b). Yoshida et al. (1994) have demonstrated that treatment with 7-nitroindazole of anesthetized rats subject to middle cerebral artery occlusion decreases the focal infarct volume in the brain, whereas the vasodilator response to topically applied acetylcholine in pial vessels and systemic blood pressure are not influenced by 7-nitroindazole. They suggested that the effectiveness of 7-nitroindazole in the doses used (25–100 mg/kg, i.p.) is due to a selective inhibition of the synthesis of NO, which is responsible for apoptosis of nerve cells in the brain, but not in the endothelium. However, the IC_{50} values of 7-nitroindazole for the rat cerebellar and bovine endothelial NOS do not differ (0.9 vs. 0.7 μM; Babbedge et al., 1993), and the hypertensive effect of 7-nitroindazole in rats under urethane anesthesia is abolished by the use of halothane (Zagvazdin et al., 1996). Recently, Reiner and Zagvazdin (1998) have summarized the discrepant data on the selectivity of 7-nitroindazole as a NOS inhibitor. The inconsistent findings may be due to studies conducted in vivo and in vitro, to the use of different anesthetics to the use of anesthetized and conscious animals and to various animal species.

The selective inhibition by 7-nitroindazole of the activities of constitutive NOS isoforms has not been evaluated in preparations that express both isoforms or in primate tissues. Therefore, the present study was undertaken to determine whether 7-nitroindazole inhibits responses mediated by NO formed by eNOS and nNOS in isolated

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monkey cerebral arteries and, if it does, whether there is a difference in the concentrations that effectively depress responses to NO released from the endothelium and from neurons. Relaxations induced by perivascular nerve stimulation by electrical pulses or nicotine are mediated by NO from the nerve innervating monkey cerebral arteries (Toda and Okamura, 1990; Toda et al., 1997), whereas those produced by histamine after treatment with cimetidine are mediated by endothelial NO in monkey and human cerebral arteries (Toda, 1990; Ayajiki et al., 1992).

2. Methods

2.1. Preparation

Japanese monkeys (*Macaca fuscata*) of either sex, weighing 6 to 10 kg, were used for the present study. The Animal Care and Use Committee of our University approved the use of monkey blood vessels.

Monkeys anesthetized with ketamine (40 mg/kg, i.m.) and sodium pentobarbital (30 mg/kg, i.m.) were killed by bleeding from the carotid arteries. The brains were removed, and the middle and posterior cerebral and basilar arteries were isolated. The arteries were helically cut into strips of approx. 20 mm in length, taking special care to preserve the endothelium. The functional integrity of the endothelium was verified by a marked relaxation (78–95%) induced by Ca^{2+} ionophore A23187 (10^{-7} M). In some of the strips, the endothelium was removed by gently rubbing the intimal surface with a cotton ball; endothelial denudation was verified by the abolishment of the response to A23187. Each specimen was vertically fixed between hooks in a muscle bath (20 ml capacity) containing modified Ringer-Locke solution of the following composition (mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0 and dextrose, 5.6. The bathing media were maintained at 37 ± 0.3 °C and aerated with a mixture of 95% O_2 and 5% CO_2; the pH of the solution was 7.38 to 7.43. The hook fixing the upper end of the strips was connected to the lever of a force-displacement transducer, and the resting tension was adjusted to 1.0 g, which is the optimal tension for inducing a maximal contraction. Before the start of experiments, the arterial strips were allowed to equilibrate in the bathing media, during which time the fluids were replaced three times about every 10 min.

2.2. Isometric tension recording

Isometric mechanical responses were displayed on an ink-writing oscillograph. The strips without endothelium were placed between stimulating electrodes (Toda et al., 1997). Electrical stimulation was applied by 0.2-ms square-wave pulses of supramaximal intensity at 2, 5 and 20 Hz for 100, 40 and 10 s, respectively. In order to analyze the effect of blocking agents, we used a stimulus frequency of 5 Hz, which produced submaximal and reproducible responses (Toda et al., 1997). The arteries were partially contracted with prostaglandin $F_{2\alpha}$ to obtain relaxation in response to electrical stimulation. In the strips with an intact endothelium contracted with prostaglandin $F_{2\alpha}$ and treated with indomethacin (10^{-6} M) and cimetidine (10^{-5} M), nicotine (10^{-4} M), histamine (5×10^{-7} M) and NO (10^{-7} M) were successively applied. Endothelium-dependent, NO-mediated relaxations are reportedly induced by histamine in monkey cerebral artery strips treated with a histamine H_2 receptor antagonist (Toda, 1990; Ayajiki et al., 1992). Papaverine (10^{-4} M) was added at the end of experiments to attain the maximal relaxation. Stimulation-induced relaxations relative to those to papaverine are presented.

2.3. Statistics and drugs used

Results shown in the text and figures are expressed as mean values ± S.E.M. Statistical analyses were made using Tukey's test after one-way analysis of variance. Drugs used were 7-nitroindazole (Lancaster Synthesis, Morecambe, UK), N^G-nitro-L-arginine (Peptide Institute, Minoh, Japan), L-arginine, nicotine (base), hexamethonium bromide (Nacalai Tesque, Kyoto, Japan), histamine hydrochloride (Kanto Chemical, Tokyo, Japan), indomethacin (Sigma, St. Louis, MO, USA), cimetidine (Fujisawa, Osaka, Japan), D-chlropheniramine maleate (Schering, Kenilworth, NJ, USA), tetrodotoxin (Sankyo, Tokyo, Japan), Ca^{2+} ionophore A23187 (Boehringer Mannheim, Mannheim, Germany), prostaglandin $F_{2\alpha}$ (Pharmacia-Upjohn, Tokyo, Japan) and papaverine hydrochloride (Dainippon, Osaka, Japan). Responses to NO were obtained by adding the $NaNO_2$ solution adjusted to pH 2 (Furchgott, 1988).

3. Results

3.1. Response to transmural electrical stimulation

In monkey cerebral artery strips without endothelium partially contracted with prostaglandin $F_{2\alpha}$, transmural electrical stimulation at 2, 5 and 20 Hz produced frequency-related relaxations, which were abolished by 3×10^{-7} M tetrodotoxin. Since the responses to 5-Hz stimulation were consistent and reproducible, this frequency was used to quantitatively analyze the action of 7-nitroindazole.

Treatment with 7-nitroindazole at 2×10^{-5} M halved the stimulation-induced relaxation and at 10^{-4} M abolished the response (Fig. 1). L-Arginine (10^{-3} M) restored the neurogenic relaxation. One example of the responses is illustrated in Fig. 2. 7-Nitroindazole at 2×10^{-5} M elicited a slight contraction ($n = 3$, $8.7 \pm 3.4\%$, $P > 0.05$, relative to contractions induced by 30 mM K^+), a relaxation ($n = 2$, 12.5 and 31.0%) or no change in tone ($n = 3$).

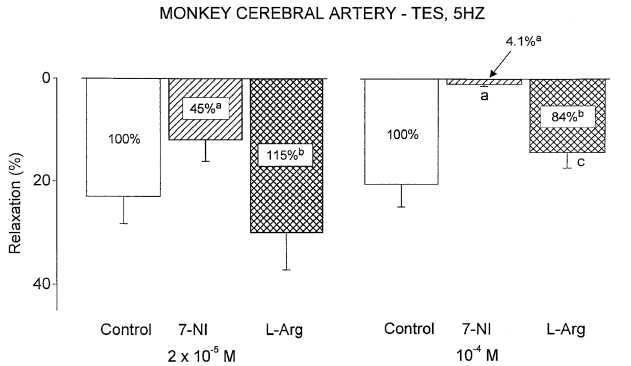

Fig. 1. Modification by 7-nitroindazole (7-NI, 2×10^{-5} and 10^{-4} M) and L-arginine (L-Arg, 10^{-3} M) of the relaxant response to transmural electrical stimulation (5 Hz) in monkey cerebral artery strips denuded of the endothelium that were partially contracted with prostaglandin $F_{2\alpha}$. Relaxations induced by 10^{-4} M papaverine were taken as 100% on the ordinate. Numbers in the columns represent values relative to those before treatment with 7-nitroindazole (control). Significantly different from control, $^{a}P < 0.01$; significantly different from the value with 7-nitroindazole, $^{b}P < 0.01$; $^{c}P < 0.05$ (Tukey's test). The 'a' and 'b' in the columns indicate statistical significance between values relative to control, and the 'a' and 'c' outside the columns indicate the difference between values relative to those for papaverine. The data were obtained with eight strips from different monkeys. Vertical bars represent S.E.M.

However, the NOS inhibitor at 10^{-4} M produced a transient contraction followed by a relaxation in two out of eight strips and only a relaxation in the remaining six; the relaxation averaged $33.6 \pm 9.6\%$ ($n = 8$) relative to that induced by 10^{-4} M papaverine.

3.2. Relaxations in response to nicotine, histamine and NO

In the arterial strips with an intact endothelium treated with 10^{-5} M cimetidine and 10^{-6} M indomethacin and partially contracted with prostaglandin $F_{2\alpha}$, the addition of nicotine (10^{-4} M), histamine (5×10^{-7} M) or NO (10^{-7} M) induced moderate relaxations (Fig. 3). The nicotine-induced relaxation was abolished by 10^{-5} M hexamethonium ($n = 6$), whereas the response to histamine was abolished by 10^{-6} M chlorpheniramine ($n = 4$), 10^{-5} M

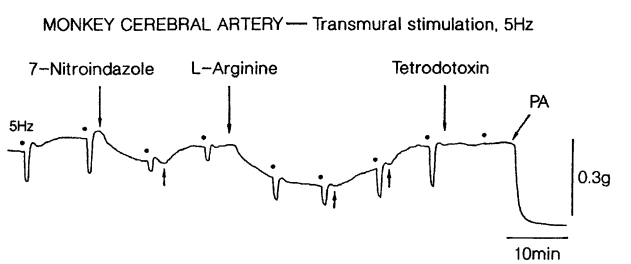

Fig. 2. A tracing of the response to transmural electrical stimulation at 5 Hz of a monkey cerebral artery strip before and after treatment with 7-nitroindazole (2×10^{-5} M), L-arginine (10^{-3} M) and tetrodotoxin (3×10^{-7} M). The strip denuded of the endothelium was partially contracted with prostaglandin $F_{2\alpha}$. PA represents 10^{-4} M papaverine, the dose that produced maximal relaxation. Upward arrows indicate the supplemental application of prostaglandin $F_{2\alpha}$ to raise arterial tone.

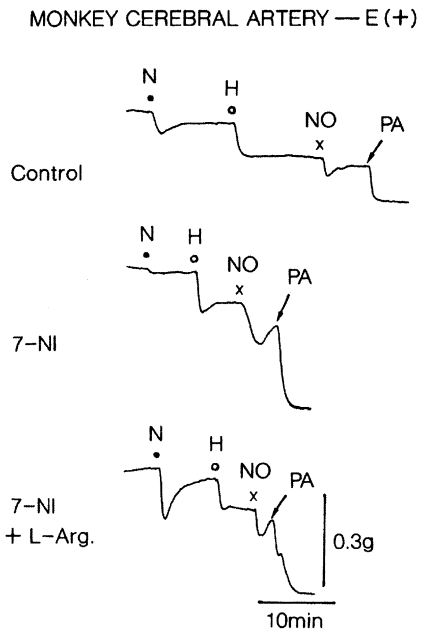

Fig. 3. Tracings of the responses to nicotine (N, 10^{-4} M), histamine (H, 5×10^{-7} M) and NO (10^{-7} M) of a monkey cerebral artery strip with endothelium before (control) and after treatment with 7-nitroindazole (7-NI, 2×10^{-5} M) and 7-nitroindazole + L-arginine (L-Arg., 10^{-3} M). The strip was partially contracted with prostaglandin $F_{2\alpha}$. PA represents 10^{-4} M papaverine, the dose that produced maximal relaxation.

N^{G}-nitro-L-arginine ($n = 4$) or by endothelium denudation ($n = 3$). Treatment with 2×10^{-5} M 7-nitroindazole attenuated the nicotine-induced relaxation by 53%, and the inhibitory effect was reversed by L-arginine (10^{-3} M) (Figs. 3 and 4). However, the responses to histamine and NO were not influenced by this concentration of 7-nitroindazole. Increasing the concentration of 7-nitroindazole to 10^{-4} M abolished the response to nicotine ($n = 4$)

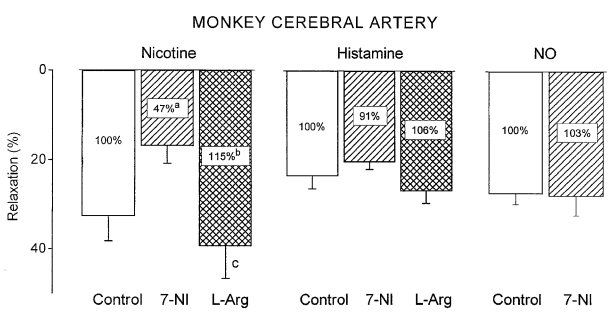

Fig. 4. Modification by 7-nitroindazole (7-NI, 2×10^{-5} M) and L-arginine (L-Arg, 10^{-3} M) of the responses to nicotine (10^{-4} M), histamine (5×10^{-7} M) and NO (10^{-7} M) in monkey cerebral artery strips denuded of endothelium and partially contracted with prostaglandin $F_{2\alpha}$. Relaxations induced by 10^{-4} M papaverine were taken as 100% on the ordinate. Numbers in the columns indicate relaxations relative to control. Significantly different from control, $^{a}P < 0.01$; significantly different from the value for 7-nitroindazole, $^{b}P < 0.01$ (comparisons of the values in the columns); significantly different from the value for 7-nitroindazole, $^{c}P < 0.05$ (comparisons of the values relative to the papaverine-induced relaxation) (Tukey's test). The data were obtained with eight strips from different monkeys. Vertical bars represent S.E.M.

and significantly inhibited the histamine-induced relaxation, from $31.2 \pm 3.8\%$ to $9.8 \pm 2.1\%$ ($n = 5$, $P < 0.01$, Tukey's test) ($69.0 \pm 6.8\%$ inhibition). The response was restored to $28.3 \pm 4.1\%$ ($89.3 \pm 7.3\%$ of control, $n = 5$, $P < 0.01$ vs. 7-nitroindazole treatment, Tukey's test) by L-arginine (10^{-3} M). The NO-induced relaxations were unaffected by 10^{-4} M 7-nitroindazole ($n = 4$).

4. Discussion

Monkey cerebral artery strips denuded of endothelium responded to transmural electrical stimulation with relaxations, which were abolished by tetrodotoxin, as demonstrated in earlier reports (Toda and Okamura, 1990; Toda et al., 1997). It is hypothesized that NO synthesized by nNOS in perivascular nerve terminals acts as a neurotransmitter in the cerebral artery (Toda and Okamura, 1992a). 7-Nitroindazole, introduced as a selective nNOS inhibitor (Moore et al., 1993a), effectively depressed the arterial response to nerve stimulation. Nicotine also produced cerebral artery relaxation, which was abolished by hexamethonium (present study) and L-arginine analogs, such as N^G-monomethyl-L-arginine and N^G-nitro-L-arginine (Toda and Okamura, 1990; Toda et al., 1997). In monkey cerebral arteries, 7-nitroindazole is about as potent as N^G-monomethyl-L-arginine and 100 times less potent than N^G-nitro-L-arginine. NOS present in perivascular nerve terminals is also activated by nicotine via an increased influx of Ca^{2+} (Toda and Okamura, 1992b; Toda et al., 1995). The inhibition by 7-nitroindazole of the nicotine-induced relaxation was identical to that of the response to electrical nerve stimulation. The inability of 7-nitroindazole to reduce the response to exogenous NO indicates that this inhibitor does not interfere with the action of NO. L-Arginine reversed the inhibitory action of 7-nitroindazole, which is not considered to be a competitive inhibitor of NOS. Similar results have been reported (Silva et al., 1995; Zagvazdin et al., 1996); however, the exact mechanism of the reversal is not known at present. The histamine-induced, endothelium-dependent relaxations in the arteries treated with cimetidine were not significantly influenced by 7-nitroindazole in a concentration (2×10^{-5} M) sufficient to halve the response to electrical stimulation or nicotine. However, increasing the concentration to 10^{-4} M, which abolished the neurogenic response, moderately inhibited the relaxation induced by histamine. In monkey and human cerebral arteries with an intact endothelium, histamine-induced relaxations are partially inhibited by cimetidine or chlorpheniramine and totally abolished by the combined treatment (Toda, 1990; Ayajiki et al., 1992). Under treatment with cimetidine, the amine-induced relaxation was abolished by N^G-nitro-L-arginine and restored by L-arginine, suggesting that histamine acts on H_1 receptor subtypes in the endothelium and activates eNOS, resulting in the release of NO. These findings led us to conclude that 7-nitroindazole preferentially inhibits nNOS in monkey cerebral arteries and that, in high concentrations, this inhibitor also inhibits eNOS. As far as the monkey cerebral arteries are concerned, 7-nitroindazole was about five times less potent in inhibiting eNOS than nNOS. According to Babbedge et al. (1993), IC_{50} values (0.9 and 0.7 μM) of 7-nitroindazole in the rat cerebellum and in bovine endothelial cells are almost identical. Whether this is due to a lack of selectivity for NOS or to the use of materials from different animal species is not known.

The addition of 7-nitroindazole at 2×10^{-5} M produced a slight contraction or a relaxation or did not alter the arterial tone, whereas N^G-nitro-L-arginine evidently contracted monkey cerebral arteries (49% at 10^{-5} M; Toda et al., 1993a), suggesting that 7-nitroindazole in this concentration inhibits, if any only slightly, the basal release of NO from the endothelium. With a higher concentration (10^{-4} M) of 7-nitroindazole, basal tone was preferentially decreased. Contractions associated with a suppression of the basal release of endothelial NO may be masked by relaxations possibly elicited by nonspecific mechanisms of 7-nitroindazole. This vasodilatation in response to 7-nitroindazole may minimize the hypertension induced by inhibition of eNOS in rats or nNOS and eNOS in monkeys and dogs (Toda et al., 1993b; Okamura et al., 1996), although the effect of 7-nitroindazole on blood pressure has not been tested in large mammals.

The present study provided evidence that 7-nitroindazole is a relatively selective nNOS inhibitor in monkey cerebral arteries. However, because the difference in effective doses of 7-nitroindazole for nNOS and eNOS was unexpectedly small, it may be difficult to choose selective doses, especially in in vivo studies.

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References

Ayajiki, K., Okamura, T., Toda, N., 1992. Involvement of nitric oxide in endothelium-dependent, phasic relaxation caused by histamine in monkey cerebral arteries. Jpn. J. Pharmacol. 60, 357–362.

Babbedge, R.C., Bland-Ward, P.A., Hart, S.L., Moore, P.K., 1993. Inhibition of rat cerebellar nitric oxide synthase by 7-nitroindazole and related substituted indazoles. Br. J. Pharmacol. 110, 225–228.

Förstermann, U., Schmidt, H.H.H.W., Pollock, J.S., Sheng, H., Mitchell, J.A., Warner, T.D., Nakane, M., Murad, F., 1991. Isoforms of nitric oxide synthase: characterization and purification from different cell types. Biochem. Pharmacol. 42, 1849–1857.

Furchgott, R.F., 1988. Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory

factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Vanhoutte, P.M. (Ed.), Vasodilatation. Raven Press, New York, NY, pp. 401–414.

Moncada, S., Higgs, A., Furchgott, R.F., 1997. XIV. International union of pharmacology nomenclature in nitric oxide research. Pharmacol. Rev. 49, 137–142.

Moore, P.K., Babbedge, R.C., Wallace, P., Gaffen, Z.A., Hart, S.L., 1993a. 7-Nitroindazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. Br. J. Pharmacol. 108, 296–297.

Moore, P.K., Wallace, P., Gaffen, Z., Hart, S.L., Babbedge, R.C., 1993b. Characterization of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: antinociceptive and cardiovascular effects. Br. J. Pharmacol. 110, 219–224.

Okamura, T., Ayajiki, K., Toda, N., 1996. Neural mechanism of pressor action of nitric oxide synthase inhibitor in anesthetized monkeys. Hypertension 28, 341–346.

Reiner, A., Zagvazdin, Y., 1998. On the selectivity of 7-nitroindazole as an inhibitor of neuronal nitric oxide synthase. Trends Pharmacol. Sci. 19, 348–350.

Silva, M.T., Rose, S., Hindmarsh, J.G., Aislaitner, G., Gorrod, J.W., Moore, P.K., Jenner, P., Marsden, C.D., 1995. Increased striatal dopamine efflux in vivo following inhibition of cerebral nitric oxide synthase by the novel monosodium salt of 7-nitro indazole. Br. J. Pharmacol. 114, 257–258.

Toda, N., 1990. Mechanisms underlying responses to histamine of isolated monkey and human cerebral arteries. Am. J. Physiol. 258, H311–H317.

Toda, N., Okamura, T., 1990. Mechanism underlying the response to vasodilator nerve stimulation in isolated dog and monkey cerebral arteries. Am. J. Physiol. 259, H1511–H1517.

Toda, N., Okamura, T., 1992a. Regulation by nitroxidergic nerve of arterial tone. News Physiol. Sci. 7, 148–152.

Toda, N., Okamura, T., 1992b. Different susceptibility of vasodilator nerve, endothelium and smooth muscle functions to Ca^{2+} antagonists in cerebral arteries. J. Pharmacol. Exp. Ther. 261, 234–239.

Toda, N., Ayajiki, K., Okamura, T., 1993a. Endothelial modulation of contractions caused by oxyhemoglobin and N^{G}-nitro-L-arginine in isolated dog and monkey cerebral arteries. Stroke 24, 1584–1589.

Toda, N., Kitamura, Y., Okamura, T., 1993b. Neural mechanism of hypertension by nitric oxide synthase inhibitor in dogs. Hypertension 21, 3–8.

Toda, N., Uchiyama, M., Okamura, T., 1995. Prejunctional modulation of nitroxidergic nerve function in canine cerebral arteries. Brain Res. 700, 213–218.

Toda, N., Ayajiki, K., Okamura, T., 1997. Inhibition of nitroxidergic nerve function by neurogenic acetylcholine in monkey cerebral arteries. J. Physiol. 498, 453–461.

Yoshida, T., Limmroth, V., Irikura, K., Moskowitz, M.A., 1994. The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. J. Cereb. Blood Flow Metab. 14, 924–929.

Zagvazdin, Y., Sancesario, G., Wang, Y.-X., Share, L., Fitzgerald, M.E.C., Reiner, A., 1996. Evidence from its cardiovascular effects that 7-nitroindazole may inhibit endothelial nitric oxide synthase in vivo. Eur. J. Pharmacol. 303, 61–69.