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7-Nitroindazole, a selective inhibitor of neuronal nitric oxide synthase: effect on sevoflurane MAC and cerebellar cyclic GMP in mice

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Abstract

Purpose. Considerable evidence suggests that nitric oxide (NO) plays a role in synaptic transmission in the central and peripheral nervous systems. However, whether inhibition of NO synthesis decreases minimum alveolar concentration (MAC) of inhalational anesthetics is controversial. We examined the effects of 7-nitroindazole (7-NI), a selective inhibitor of neuronal NOS (nNOS), on the MAC of sevoflurane and cerebellar cyclic guanosine monophosphate (cGMP) levels in mice.

Methods. Sevoflurane MAC and cerebellar cGMP levels were determined in mice after acute intraperitoneal or week-long gavage feeding of 7-NI. Sevoflurane MAC and cerebellar cGMP levels after chronic treatment were measured on days 1, 4, and 7 and were repeated after an acute intraperitoneal dose of nitro^{G}-L-arginine methylester (L-NAME).

Results. Acute and chronic treatment with 7-NI decreased the sevoflurane MAC by 20%–30%. Reduction of cerebellar cGMP levels was greater after intraperitoneal administration of NOS inhibitors than after gavage feeding of 7-NI.

Conclusion. Acute or chronic selective inhibition of neuronal NOS decreases the sevoflurane MAC and cerebellar cGMP levels in mice. 7-NI permitted probing of the role of NO in perception of noxious stimuli.

Kew words: Potency, MAC, Mouse, Nitric oxide, Nitric oxide synthase, NOS inhibitor, Volatile anesthetics, Sevoflurane

Introduction

Nitric oxide (NO), produced enzymatically by nitric oxide synthase (NOS) from L-arginine, exerts many of its effects by increasing the intracellular concentrations of cyclic guanosine monophosphate (cGMP) in target cells through activation of soluble guanylate cyclase (sGC). There are at least three homologous forms of NOS in the central nervous system (CNS) [1–3]. The neuronal form of NOS (nNOS) is most abundant and localizes mainly to neurons, whereas the endothelial and inducible forms are present predominantly in vascular endothelium and in astrocytes and microglia, respectively [4,5].

Although evidence suggests that the L-arginine–NO cGMP pathway plays an important role in nociception [6–8], whether inhibition of NO synthesis decreases the minimum alveolar concentration (MAC) of inhalational anethetics is controversial. In the rat, administration of nitro^{G}-L-arginine methylester (L-NAME) has been reported to reduce or not change the halothane MAC [9,10]. Recently, we have demonstrated that isoflurane MAC is decreased by NOS inhibition with L-NAME in wild-type mice but is preserved in mice that congenitally lack the neuronal NOS gene [11]. Others have reported that chronic NOS inhibition with L-NAME decreased cerebellar NOS activity and cGMP levels without changing the halothane MAC in rats [12]. We postulated that these conflicting results may be due, at least partly, to the difference between chronic and acute NOS inhibition and the isoform nonselective nature of NOS inhibition with L-NAME. 7-Nitroindazole (7-NI) is a potent neuronally selective NOS inhibitor. An acute treatment with 7-NI has been reported to be antinociceptive in mice and reduces the isoflurane MAC in rats [13,14]. Furthermore, unlike previously studied nonselective NOS inhibitors, 7-NI does not increase arterial blood pressure [13,14].

In the present study we examined the effects of NOS inhibition on the sensitivity to inhalational anesthetics by studying the effects of acute or chronic administration of 7-NI on the sevoflurane MAC and cerebellar cGMP levels in mice. The MAC determination was repeated after acute and week-long 7-NI administration.

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Materials and methods

Altogether 144 male cd-1 mice weighing 28–33 g were used. The studies were approved by the institutional committee on animal research of Teikyo University School of Medicine. All animals were housed in a room with controlled temperature (24° ± 1°C), humidity, and artificial light. The mice had free access to food and water and were tested after a minimum of 4 days of acclimation to the housing condition. We suspended the 7-NI in arachis oil by sonication (Sonifier, Branson, Danbury, CT, USA) and administered it to mice in a volume of 4 ml/kg. 7-NI and L-NAME were obtained from Sigma (St. Louis, MO, USA).

Acute protocol

In acute experiments mice were given 7-NI 120 mg·kg^{-1}, L-arginine 600 mg·kg^{-1}, or the same volume of arachis oil intraperitoneally (IP). The sevoflurane MAC and cerebellar cGMP level were determined approximately 60 min after drug administration. A total of 80 mice were used for the acute experiments; 16 mice were used in each treatment group: 8 mice for MAC determination and 8 mice for cGMP determination. We chose the 7-NI dose based on previous studies [13,14].

Chronic protocol

In chronic experiments we administered 7-NI 120 mg·kg^{-1} or the same volume of arachis oil by oral gavage feeding every 12 h for 7 consecutive days. Altogether 64 mice were used for the chronic experiments. Determination of the sevoflurane MAC and cerebellar cGMP levels were performed at the following time points (n = 8 in each group): baseline, 4 days prior to the start of the experiment; day 1, 60 min after the first gavage feeding on the first day; day 4, 60 min after the first gavage on the fourth day; day 7, 60 min after the last gavage on the seventh day. In additional experiments, L-NAME 50 mg·kg^{-1} was acutely given to 8 mice after a week of 7-NI gavage, and the sevoflurane MAC and cGMP levels were determined.

Determination of MAC baseline values in mice

Baseline values of the sevoflurane MAC were established according to methods described previously [11,15–17]. Briefly, groups of eight mice were placed in individual acrylic cylinders (15 cm long, 5 cm in diameter) for the determination of MAC values. Each cylinder was fitted with a rubber stopper at one end through which the mouse's tail and a rectal temperature probe protruded. The temperature was monitored and maintained between 36.5° and 38.0°C with a warming lamp. A gas sample was continuously drawn from the expiratory limb of the circuit, and the anesthesia concentration was measured with an infrared analyzer (Datex Ultima, Helsinki, Finland). Mice initially breathed approximately 4.0% sevoflurane in oxygen (4 l·min^{-1} total gas flow) for 30 min. A tail clamp (alligator clip) was applied to the tail for 1 min, and the mice were observed for movement in response to the stimulation. In each case the tail was stimulated proximal to the previous test site. Only the middle third of the tail was used for tail-clamping. The concentration of the anesthetic agent at which the mouse exhibited motor activity (gross movements of the head, extremities, body) was considered one that permitted a positive motor response. The anesthetic concentration was increased (or decreased) in steps of 0.3%–0.4% until the positive response disappeared (or vice versa), with 15 min allowed for equilibration after each change of anesthetic concentration. MAC is defined as the concentration midway between the highest concentration that permitted movement in response to the stimulus and the lowest concentration that prevented movement. A typical MAC study of a group of eight mice took approximately 3–4 h including the initial equilibration period.

Measurement of cerebellar cGMP levels

The mice were sacrificed by head-focused microwave irradiation at 9 kW for 0.35 s (NJE 2204; New Japan Radio, Tokyo, Japan) [18]. The cerebella were dissected out, weighed, and rapidly frozen. The frozen tissue was homogenized in ice-cold 8% trichloroacetate and centrifuged at 15000 g for 15 min at 4°C. The supernatants were washed three times with water saturated with diethylether and then assayed in duplicate for cGMP using a cGMP Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Blood gas analysis

To rule out the presence of hypoxia, hypercapnia, and acidosis during these experiments, we sampled arterial blood from four mice in each group. To this end, percutaneous left ventricular puncture was performed at the end of the MAC determination.

Statistics

All values were expressed as the mean ± SEM. Statistical analysis was performed using one- or two-way analysis of variance (ANOVA) followed by a multiple comparisons test (Tukey-Kramer). $P < 0.05$ was assumed significant.

Results

The baseline value of the sevoflurane MAC in mice was 3.28 ± 0.06%. Acute intraperitoneal administration of 7-NI 120 mg·kg^{-1} or L-NAME 50 mg·kg^{-1} decreased the sevoflurane MAC to 2.64 ± 0.11% ($P < 0.01$) and 2.30 ± 0.09% ($P < 0.01$), respectively; these reductions were completely reversed by administration of L-arginine 600 mg·kg^{-1} IP (Table 1). An intraperitoneal administration of arachis oil 4 ml/kg or L-arginine 600 mg·kg^{-1} per se did not affect the sevoflurane MAC (Table 1).

Week-long administration of 7-NI by oral gavage caused no apparent abnormal behaviors in mice. The sevoflurane MAC in mice at baseline and on days 1, 4, and 7 were 3.29 ± 0.08%, 2.58 ± 0.14% ($P < 0.01$), 2.76 ± 0.10% ($P < 0.05$), and 2.81 ± 0.09% ($P < 0.05$), respectively (Fig. 1A). Gavage feeding with arachis oil for a week caused no significant changes in the sevoflurane MAC (Fig. 1A,B).

The cerebellar cGMP level in the control mice was 214.5 ± 24.9 pmol/g tissue. Acute administration of 7-NI 120 mg·kg^{-1} IP or L-NAME 50 mg·kg^{-1} IP decreased cerebellar cGMP levels to 7.8 ± 0.9 pmol/g tissue ($P < 0.01$) and 6.4 ± 0.8 pmol/g tissue ($P < 0.01$), respectively. The cerebellar cGMP levels during a week-long treatment with 7-NI (120 mg·kg^{-1}) at baseline and on days 1, 4, and 7 were 214.5 ± 24.9, 93.5 ± 17.8 ($P < 0.01$), 84.4 ± 18.4 ($P < 0.01$), and 114.5 ± 18.0 ($P < 0.01$) pmol/g tissue, respectively (Fig. 1B). In control experiments, a week-long gavage of arachis oil failed to alter cerebellar cGMP in mice (Fig. 1B).

Acute administration of L-NAME 50 mg·kg^{-1} IP after a week of 7-NI gavage feeding decreased the cerebellar cGMP level to 3.7 ± 0.6 pmol/g tissue without further reducing sevoflurane MAC (2.77 ± 0.10%) (Fig. 1A,B). In contrast, acute intraperitoneal administration of L-NAME after a week of arachis oil gavage feeding significantly decreased both the cerebellar cGMP levels (6.4 ± 0.8 pmol/g tissue) and the sevoflurane MAC (2.30 ± 0.09%).

The results of blood gas analysis at the end of MAC experiments after a week of 7-NI administration showed no significant difference between groups (Table 2).

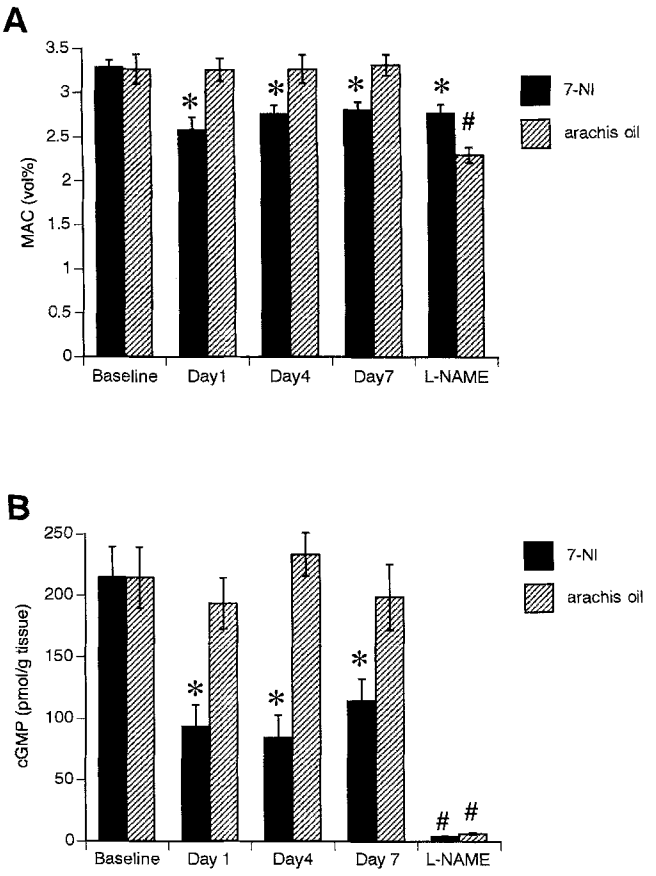

Fig. 1. Influence of a week-long gavage feeding of 7-NI (120 mg·kg^{-1} q12 h, days 1, 4, 7), arachis oil (4 ml/kg q12 h, days 1, 4, 7), and acute administration of L-NAME (50 mg/kg IP, day 7) on the sevoflurane MAC (**A**) and cerebellar cGMP levels (**B**) in mice. Values are means ± SE ($n = 8$). *Value differs significantly from baseline and from arachis oil ($P < 0.05$). #Value significantly differs from baseline ($P < 0.05$)

Table 1. Effects of acute intraperitoneal or oral gavage administration of 7-NI, L-NAME, L-arginine, and arachis oil on sevoflurane MAC and cGMP concentration in mice ($n = 8$)

Parameter	Baseline	7-NI (120 mg/kg) IP	7-NI (120 mg/kg) PO	L-NAME (50 mg/kg IP)	7-NI (120 mg/kg IP) + L-arginine (600 mg/kg IP)	Arachis oil (4 ml/kg IP)	L-Arginine (600 mg/kg IP)
MAC (vol%)	3.28 ± 0.06	2.64 ± 0.11**	2.58 ± 0.14**	2.30 ± 0.09**	3.36 ± 0.23	3.34 ± 0.13	3.30 ± 0.12
cGMP (pmol/g tissue)	214.5 ± 24.9	7.8 ± 0.9**	93.5 ± 17.8*	6.4 ± 0.8**	261 ± 22.0	240 ± 26.3	260 ± 28.9

7-NI, 7-nitroindazole; L-NAME, nitro^{G}-L-arginine methylester; MAC, minimum alveolar concentration; cGMP, cyclic guanosine monophosphate.

*Value differs significantly from baseline ($P < 0.01$. **Value differs significantly from baseline ($P < 0.001$).

Table 2. Results of arterial blood gas analyses at the conclusion of the MAC experiments ($n = 8$)

Analysis	Arachis oil (4 ml/kg)	7-NI (120 mg/kg)	7-NI (120 mg/kg) + L-arginine (600 mg/kg)
pH_a	7.50 ± 0.02	7.49 ± 0.01	7.41 ± 0.01
PaO_2 (mmHg)	126.3 ± 12.3	159.2 ± 25.4	135.8 ± 9.0
$PaCO_2$ (mmHg)	30.8 ± 1.6	31.8 ± 1.7	33.8 ± 1.4

There were no significant differences between the groups.

Discussion

The present study reveals that acute or chronic administration of the neuronally selective NOS inhibitor 7-NI decreases the MAC of sevoflurane and the cerebellar cGMP levels in the mouse. During the week-long 7-NI gavage feeding, however, reduction of the sevoflurane MAC and cerebellar cGMP levels reached a plateau after day 1, and no further reduction of either parameter was observed. These results suggest that inhibition of neuronal NOS and the resultant reduction of cGMP levels in the brain are associated with a state of greater sensitivity to sevoflurane anesthesia. These observations confirmed the results of our previous studies in which the isoflurane MAC was reduced in wild-type mice only after acute intraperitoneal administration of L-NAME but not after chronic L-NAME feeding or in neuronal NOS knockout mice [11].

The cerebellar cGMP values at baseline and after NOS inhibition in the present study are consistent with those reported in the literature [12,19]. Although acute intraperitoneal administration of 7-NI or L-NAME profoundly reduced cerebellar cGMP levels, chronic gavage feeding of 7-NI only moderately reduced cGMP levels in mice. In contrast, the magnitude of MAC reduction was similar in both groups of mice (Fig. 1). This discrepancy suggests the presence of ceiling effects of NOS inhibition and the resultant reduction of cGMP by the reduction of the MAC. Although we did not examine the dose–response relation between 7-NI and sevoflurane MAC in the present study, similar ceiling effects of NOS inhibitors on MAC have been reported [9,11,14]. These observations are consistent with the widely accepted notion that multiple pathways coexist to mediate nociception or consciousness. Therefore, it is likely that NOS inhibition and the resultant decrease in cGMP level do not reduce sevoflurane MAC beyond a certain degree because of coexisting compensatory pathways.

Other possible explanations for this discrepancy were considered. It is possible that the cerebellar NOS activity and cGMP levels may not reflect those of the true sites of nociception and consciousness in the CNS. Although the mechanisms of general anesthesia are largely unknown at present, there is evidence that the response to the tail clamp is a spinally mediated response. It was reported that rats that underwent precollicular decerebration or spinal cord transection had no change in isoflurane MAC, and goats that had preferential delivery of isoflurane to the brain had exaggerated anesthetic requirements [20,21]. These results suggest that the spinal cord is an important site of anesthetic action.

Alternatively, MAC sparing effects of NOS inhibitors may not be mediated via the sGC-cGMP mechanism. It has been well documented that NO has various biological roles that are mediated in a cGMP-independent fashion. For instance, NO has been shown to interact directly and indirectly with various inhibitory neurotransmitters such as GABA, glycine, opioid, and muscarinic receptors [22,23]. Although the significance of these interactions on the mechanism of general anesthesia is unclear, it is conceivable that NOS inhibitors modify sensitivity to general anesthesia based on these mechanisms. If this is the case, cGMP levels in the CNS may not necessarily correlate with the reduction of MAC produced by inhibition of neuronal NOS.

The fact that sevoflurane MAC and cerebellar cGMP levels were only moderately decreased after 7 days of 7-NI feeding suggests that NO derived from other NOS isoforms are compensating for the lack of neuronal NOS-derived NO. To explore this possibility, we have examined effects of acute intraperitoneal L-NAME administration after chronic 7-NI treatment, as L-NAME nonselectively inhibits all isoforms of NOS. Our observation that acute L-NAME after a week of 7-NI significantly decreased cerebellar cGMP without further reducing sevoflurane MAC suggests that the remaining cGMP may be derived from activities of other NOS isoforms, but the MAC-reducing effects of NOS inhibition are saturated, regardless of the NOS isoform inhibited. Our results further suggest that there is an apparent discrepancy between MAC and cerebellar cGMP levels after NOS inhibition. To clarify the relations between the two parameters, additional studies examining full dose–response relations are needed.

We have previously reported that the isoflurane MAC value returned to its baseline after a week of L-NAME gavage feeding, possibly because of the

development of compensatory mechanisms [11]. However, after a week of 7-NI treatment, the sevoflurane MAC did not return to its baseline value in the present study. It is possible that the development of compensation was not as robust as after a week of L-NAME, as 7-NI only selectively inhibits neuronal NOS. It is more likely that nNOS inhibition with chronic 7-NI was not as complete owing to the relatively short half-life of 7-NI, especially when given orally as reported previously for rats [24]. The time course of neuronal NOS inhibition and the development of compensation remains unclear.

In conclusion, the present study demonstrates that acute or chronic treatment with 7-NI reduces the sevoflurane MAC and cerebellar cGMP levels in mice. The correlation between the sevoflurane MAC and cerebellar cGMP level was not linear, and a dissociation between the magnitude of the reduction of both parameters after the treatment with NOS inhibitors suggests a ceiling effect. The precise mechanism and the time course of the effects of NOS inhibition on the anesthetic potency of general anesthetics remains to be determined.

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