Anesthesiology
1998; 89:143–8

Lack of Correlation between the Reduction of Sevoflurane MAC and the Cerebellar Cyclic GMP Concentrations in Mice Treated with 7-Nitroindazole

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Background: **Although inhibition of nitric oxide synthase (NOS) has been reported to be antinociceptive and to reduce the threshold of general anesthesia, the mechanism of action is largely unknown. Specifically, the relation between the minimum alveolar concentration (MAC)-reducing effects of NOS inhibition and cyclic guanosine monophosphate (cGMP) concentrations in the brain has not been defined. To further characterize the effects of NOS inhibition, the authors studied the relation between the MAC of sevoflurane and the cGMP concentration of the brain after acute and chronic treatment with a neuronally selective NOS inhibitor, 7-nitroindazole (7-NI).**

Methods: **Sevoflurane MAC and cerebellar cGMP concentrations were determined in mice after acute intraperitoneal administration or after 1, 2, 3, and 4 days of gavage feeding of 7-NI. After acute or chronic treatment with 7-NI, the mice were separated into two groups. Sevoflurane MAC was measured by a tail-clamp method in the first group, and cerebellar cGMP concentrations were measured by enzyme-linked immunosorbent assay in the second group of the mice.**

Results: **In mice, acute intraperitoneal administration of 7-NI dose dependently decreased sevoflurane MAC and cerebellar cGMP; and 4-day-long gavage feeding with 7-NI (500 mg/kg, every 8 h) time dependently decreased cerebellar cGMP, but sevoflurane MAC was reduced only for the first 2 days and returned to its baseline after 3 days of 7-NI feeding.**

Conclusions: **Although an acute selective inhibition of neuronal NOS decreases sevoflurane MAC and cerebellar cGMP concentrations in mice, there was a dissociation between the two parameters during long-term neuronal NOS inhibition. There may be cGMP-independent compensatory mechanisms that mediate nociception when NOS is chronically inhibited. (Key words: Minimum alveolar concentration; nitric oxide; nitric oxide synthase inhibitor; potency; sevoflurane; volatile anesthetics; 7-nitroindazole.)**

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

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Received from the Departments of Anesthesiology and Clinical Research, Teikyo University School of Medicine, Ichihara Hospital, Chiba, Japan. Submitted for publication January 8, 1997. Accepted for publication March 6, 1998. Supported in part by a grant from Sasagawa Foundation, Tokyo, Japan, and from Daido-Hoxan Co., Tokyo, Japan.

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NITRIC oxide is produced enzymatically by nitric oxide synthase (NOS) from L-arginine and exerts many of its effects by increasing intracellular concentrations of cyclic guanosine monophosphate (cGMP) in target cells by activating soluble guanylate cyclase. There are at least three homologous forms of NOS in the central nervous system.[1–5] The neuronal form of NOS (nNOS or NOS1) is most abundant and localizes primarily to neurons, whereas the endothelial and inducible forms are present predominantly in vascular endothelium and in astrocytes and microglia, respectively.[6–8]

Research has shown that NO mediates the cerebellar increase of cGMP concentration in response to stimulation by N-methyl-D-aspartate,[3–5] glutamate,[4] and kainate.[9] On the other hand, halothane[10] and enflurane[11] decrease the cGMP concentration of selected brain regions, including the cerebellum. Recently, administration of nitro^{G}-L-arginine methylester, a nonselective NOS inhibitor, was reported to be antinociceptive and reduces the threshold of anesthesia in rats and mice.[12–14] Although these and other evidence suggest an im-

portant role of the NO-cGMP system in nociception and anesthesia, the mechanism of reduction of the MAC after NOS inhibition is unknown. Furthermore, Adachi *et al.*[15,16] reported conflicting data by showing that chronic treatment with nitro^{G}-L-arginine methylester reduced NOS activity and cGMP concentrations without changing the halothane MAC in rats, and they suggested that there was no relation between NOS activity and the MAC of volatile anesthetics. Therefore, whether inhibition of NOS1 (acute or chronic) decreases the MAC of volatile anesthetics and cerebellar cGMP concentrations is still controversial.

To better characterize the mechanism of action of the MAC-reducing effects of NOS inhibitors, and to discover the neurobiological significance of cerebellar cGMP concentrations, we studied the relation between the MAC for sevoflurane and the cGMP concentration of the brain after administration of a selective inhibitor of NOS1. We specifically designed these experiments to address the following questions: (1) Does acute or chronic selective inhibition of NOS1 with 7-nitroindazole (7-NI) reduce sevoflurane MAC and cerebellar cGMP concentrations in mice? (2) If it does, what is the relation between the two parameters?

Materials and Methods

One hundred sixty male cd-1 mice (28–33 g; SEASCO, Saitama, Japan) 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 used after a minimum of 4 days' acclimation to the housing condition. Each mouse underwent only one determination of either MAC or cerebellar cGMP.

Determination of Baseline Values of the Minimum Alveolar Concentration in Mice

Baseline values of the MAC of sevoflurane were established according to the methods described previously.[14,17–19] Briefly, groups of eight mice were placed in individual acrylic cylinders (15 cm long and 5 cm in diameter) to determine 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°C with a warming lamp. A gas sample was drawn continuously from the outflow tube from the cylinder, and the anesthetic concentration was measured with an infrared analyzer (Datex Ultima, Helsinki, Finland). Mice initially breathed approximately 4% sevoflurane in oxygen (4 l/min 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 every 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, or all three) was considered one that permitted a positive motor response. The anesthetic concentration was increased (or decreased) in steps of 0.3% to 0.4% until the positive response disappeared (or vice versa), with 15 min for equilibration allowed after each change of anesthetic concentration.[20,21] The 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 h, including the initial equilibration period.

Measurement of Cerebellar Cyclic Guanosine Monophosphate Concentrations

To determine cerebellar cGMP concentrations, the mice were killed by head-focused microwave irradiation at 9 kW for 0.35 s (NJE 2204; New Japan Radio Co., Tokyo, Japan).[22] The cerebella were dissected, weighed, and rapidly frozen. The frozen tissue was homogenized in ice-cold 8% trichloroacetate and centrifuged at 15,000*g* for 15 min at 4°C. The supernatants were washed three times with water saturated with diethyl ether and then assayed in duplicate for cGMP using a cGMP enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). The detection limit of cGMP was 0.09 pmol/ml. All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Acute Protocol. The effects of six doses of 7-NI (20, 60, 80, 120, 500, and 1,000 mg/kg given intraperitoneally) on sevoflurane MAC and cerebellar cGMP concentrations were examined in 96 mice. After administration of 7-NI, mice were separated into two groups of 48 mice each, and the mice in the first group were used for cGMP determination and the mice in the second group were used for MAC determination. Sevoflurane MAC was determined in at least eight mice after intra-

peritoneal administration of each dose of 7-NI. In another 48 unanesthetized mice, the cerebellar cGMP concentration was determined approximately 60 min after intraperitoneal administration of 7-NI. The 7-NI was suspended in arachis oil by sonication (Sonifier, Branson, Danbury, CT) on ice immediately before administration, and it was administered intraperitoneally to mice in a volume of 4 ml/kg. We examined the reversibility of 7-NI–induced reduction of sevoflurane MAC by administering L-arginine. After sevoflurane MAC was determined in a group of mice pretreated with 7-NI (500 mg/kg), L-arginine (600 mg/kg) was dissolved in 4 ml/kg normal saline and injected intraperitoneally. The sevoflurane MAC was redetermined beginning 15 min later. As control, effects of intraperitoneal administration of arachis oil and L-arginine on sevoflurane MAC were also examined in eight mice each.

Long-Term Experiments. In long-term experiments, we administered 500 mg/kg 7-NI in a volume of 2 ml/kg or the same volume of arachis oil to 64 mice by oral gavage feeding every 8 h for 4 consecutive days. Mice were separated into two groups of 32 mice each, and determination of sevoflurane MAC in the first group of mice was begun immediately after the last gavage feeding on days 1 through 4. The other 32 mice were killed 60 min after the last gavage feeding on days 1 through 4.

Blood Gas Analysis

To rule out the presence of hypoxia, hypercapnia, and acidosis during these experiments, we sampled arterial blood from all mice in the acute protocol, by percutaneous left ventricular puncture at the end of the MAC determination.

Statistics

All values were expressed as means ± SD. The statistical analysis was performed using one- or two-way analysis of variance followed by a multiple comparisons test (Tukey-Kramer or Dunnet). The Spearman correlation coefficient was calculated between the values of sevoflurane MAC and cerebellar cGMP concentrations. $P < 0.05$ was considered to be significant.

Results

The baseline value of sevoflurane MAC in mice was 3.22 ± 0.38%. Acute intraperitoneal administration of 7-NI from 20 to 1,000 mg/kg modestly decreased sevoflurane MAC in mice (fig. 1). The reduction of sevoflurane MAC was completely reversed by intraperitoneal administration of 600 mg/kg L-arginine. Intraperitoneal administration of 4 ml/kg arachis oil or 600 mg/kg L-arginine *per se* did not affect sevoflurane MAC (table 1).

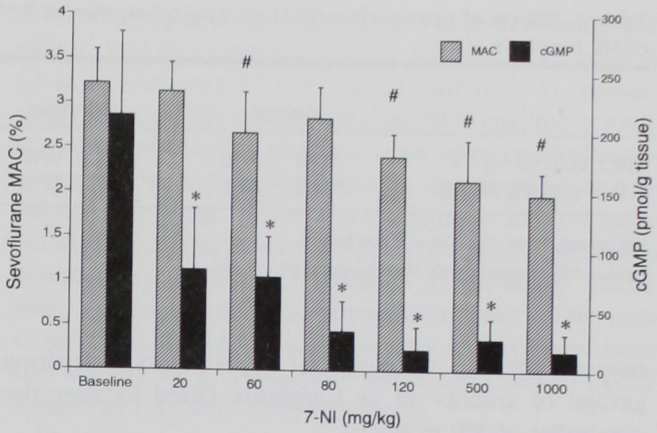

Fig. 1. Influence of acute intraperitoneal administration of 7-nitroindazole (20, 60, 80, 120, 500, and 1,000 mg/kg) on the sevoflurane minimum alveolar concentration and cerebellar cyclic guanosine monophosphate levels in mice. Values are presented as mean ± SD (n = 8 per group). Bars denoted by * and # are significantly different from baseline ($P < 0.05$).

The sevoflurane MACs in mice at baseline and days 1 to 4 were 3.22 ± 0.38%, 2.68 ± 0.39% ($P < 0.05$), 2.71 ± 0.52% ($P < 0.05$), 2.94 ± 0.49%, and 2.88 ± 0.52%, respectively (fig. 2). Although administration of 7-NI by oral gavage every 8 h for 4 days caused no apparent abnormal behaviors or distress in most of the mice, two died on day 3 and another mouse died on day 4 of undetermined causes. Gavage feeding with arachis oil or L-arginine for 1 week caused no significant changes in sevoflurane MAC.

The cerebellar cGMP concentration in the control mice was 214.5 ± 70.4 pmol/g tissue. Acute intraperitoneal administration of 7-NI from 20 to 1,000 mg/kg dose dependently decreased cerebellar cGMP concentrations to 16.9 ± 14.5 pmol/g tissue at 1,000 mg/kg ($P < 0.01$; fig. 1). A high correlation coefficient was found between the values of sevoflurane MAC and the cerebellar cGMP concentrations after an acute intraperitoneal administration of 7-NI ($r = 0.929$, $P < 0.01$). The cerebellar cGMP concentrations during 4-day-long treatment with 7-NI (500 mg/kg) at baseline and on days 1 to 4 were 214.5 ± 70.4 pmol/g, 121.5 ± 19.7 pmol/g ($P < 0.01$), 60 ± 33.2 pmol/g ($P < 0.01$), 57 ± 30.3 pmol/g ($P < 0.01$), and 47.3 ± 14.9 pmol/g ($P < 0.01$) tissue,

Table 1. Effects of Intraperitoneal (i.p.) Administration of 7-NI, L-Arginine, and Arachis Oil on Sevoflurane MAC and Cerebellar cGMP Levels in Mice

	Baseline	7-NI 500 mg/kg	L-Arginine 600 mg/kg	Arachis Oil 4 ml/kg	7-NI 500 mg/kg + L-Arginine 600 mg/kg
MAC (volume %)	3.22 ± 0.38	2.14 ± 0.42*	3.40 ± 0.88	3.32 ± 0.93	3.06 ± 0.34
cGMP (pmol/g tissue)	214.5 ± 70.4	26.7 ± 17.5*	244.0 ± 80.6	228.6 ± 76.4	251.0 ± 67.9

Values are mean ± SD (n = 8 per group).

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

respectively (fig. 2). In control experiments, 4-day-long gavage of arachis oil or L-arginine failed to alter the cerebellar cGMP in mice.

The results of blood gas analysis at the end of MAC experiments after 7-NI administration showed no significant difference between groups.

Discussion

Our results showed in mice that (1) acute intraperitoneal administration of 7-NI dose dependently decreased sevoflurane MAC and cerebellar cGMP; and (2) 4-day-long gavage feeding with 7-NI time dependently decreased cerebellar cGMP, but sevoflurane MAC was reduced only for the first 2 days and returned to its baseline after 3 days of 7-NI feeding. These observations confirmed the results of our previous studies in which isoflurane MAC was reduced in wild-type mice only after acute intraperitoneal administration of nitro^{G}-L-arginine methylester, but not after chronic nitro^{G}-L-arginine methylester feeding or in NOS1 knockout mice.[14] Therefore, together with our previous findings, our current results support the hypothesis that the MAC of volatile anesthetics is decreased by acute NOS1 inhibition but not by long-term deficiency of NOS1 activity, which also suggests the presence of other compensatory mechanisms that mediate nociception when NOS1 is chronically inhibited.

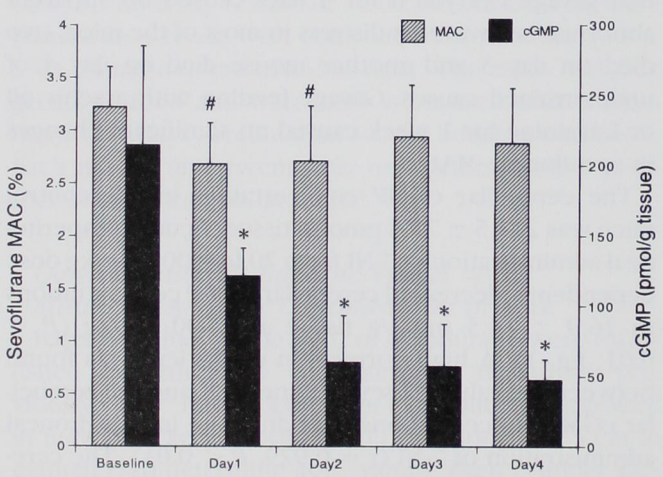

Fig. 2. Influence of 4-day-long gavage feeding of 7-nitroindazole (500 mg/kg given every 8 h) on the sevoflurane minimum alveolar concentration and cerebellar cyclic guanosine monophosphate levels in mice. Values are presented as means ± SD (n = 8 per group). Bars denoted by * and # are significantly different from baseline ($P < 0.05$).

The effects of various pharmacologic agents and other variables on cGMP concentrations in the central nervous system have been studied extensively.[10,11,23–25] Agonists of excitatory amino acid receptors enhance basal cGMP concentrations,[4,5,8,9,23] but most central nervous system depressants such as excitatory amino acids–antagonists,[26,27] GABAergic agonists,[28] barbiturates,[10,29] and volatile anesthetics[10,11] decrease basal cGMP concentrations and block increases in cGMP concentrations. Nitric oxide synthase inhibitors have also been shown to decrease basal cGMP concentrations *in vivo*[16,23] at doses that reduce MAC for volatile anesthetics. Although these data suggest that NOS inhibitors may be central nervous system depressants, quantitative and temporal relations between the MAC-reducing and cGMP-reducing effects of NOS inhibitors have not been examined carefully.

Although there was a marked difference in the magnitude of reduction of the values of sevoflurane MAC and cerebellar cGMP concentrations, we found a high correlation coefficient between the two parameters after acute administration of 7-NI. We measured sevoflurane MAC and cerebellar cGMP in two separate groups of mice after 7-NI treatment. Therefore, regardless of whether they were used for MAC or cGMP determination, we can assume that the cGMP values we measured represent the post–7-NI cGMP concentrations in all the mice. Although a high correlation coefficient itself does not imply a direct relation between the MAC-

reducing effects of 7-NI and cerebellar cGMP concentrations, our results show a quantitative correlation between the two parameters.

During long-term 7-NI treatment, however, there was no correlation between sevoflurane MAC and baseline cerebellar cGMP concentrations. Although the cGMP concentrations were time dependently depressed for 4 days by the long-term 7-NI treatment, a relatively minor reduction of sevoflurane MAC was observed only for 2 days, and after 3 days MAC was no longer reduced by 7-NI. The decreased cGMP concentrations indicated that NOS1 activity was actually inhibited by 7-NI, confirming the results of previous studies.[30] The observation that sevoflurane MAC was reduced only after acute but not after long-term 7-NI treatment confirmed the results of previous studies in which long-term nitro^{G}-L-arginine methylester failed to reduce MAC of volatile anesthetics in wild-type mice,[14,16] and normal anesthetic sensitivity was preserved in the NOS1 knockout mouse.[14] We speculated that alternative neural mechanisms compensated for the lack of NO in the brain, and the normal sensitivity to volatile anesthetics was preserved in these animals.

It is possible that the MAC-reducing effects of NOS inhibitors may not be mediated by the soluble guanylate cyclase-cGMP mechanism. It has been well documented that NO has various biological roles that are mediated in a cGMP-independent manner. For example, NO has been shown to interact directly and indirectly with various inhibitory neurotransmitters such as GABA, glycine, opioid, and muscarinic receptor mechanisms.[31,32] Although the significance of these interactions on the mechanism of general anesthesia is unclear, it is conceivable that NOS inhibitors modify the sensitivity to general anesthesia based on these or other cGMP-independent mechanisms. If this is true, cGMP concentrations in the central nervous system may not necessarily correlate with the reduction of MAC produced by NOS1 inhibition.

Although the mechanisms of general anesthesia are largely unknown, evidence suggests that the response to the tail clamp is spinally mediated. 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.[33-35] These results suggest that the spinal cord is an important site of anesthetic action. On the other hand, research has shown that administration of 7-NI (25 mg/kg given intraperitoneally) to conscious mice resulted in a paralleled reduction in both cerebellar and spinal cord NOS enzyme activity monitored *ex vivo* of 63.4 ± 2.6% and 53.1 ± 3.8% (both n = 4), respectively.[36] Cerebellar and spinal cord NOS enzyme activity showed very similar changes even after coadministration of 7-NI and flurbiprofen, a nonsteroidal anti-inflammatory drug.[36] Therefore, changes in cerebellar cGMP concentrations might closely reflect changes in spinal cord cGMP concentrations in our study. Therefore, we believe that measuring cerebellar cGMP concentrations would give us important information about the activity of a NO-soluble guanylate cyclase-cGMP system in the nociceptive pathway.

In conclusion, we found that acute treatment with 7-NI reduces sevoflurane MAC and cerebellar cGMP concentrations in a dose-dependent manner in mice. Our results also showed a dissociation between both parameters during chronic NOS1 inhibition, suggesting that the MAC-reducing effects of 7-NI may not be mediated *via* the soluble guanylate cyclase-cGMP system, or that there are cGMP-independent compensatory mechanisms that mediate nociception during long-term NOS1 inhibition. The precise mechanism and the time course of the effects of NOS inhibitors on the anesthetic potency of general anesthetics remains to be determined.

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