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General anesthetics inhibit the nitrous-oxide-induced activation of corticotropin releasing factor containing neurons in rats

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Abstract

The activation of intracerebral corticotropin releasing factor (CRF) system is involved in nitrous oxide analgesia. We evaluated the effect of general anesthetics on nitrous-oxide-induced CRF activation and antinociception. Male Sprague–Dawley rats inhaled isoflurane (0%, 0.6%, 1.0% and 1.5%) or were administered with intravenous propofol (0, 0.1 and 0.2 mg/kg/min), with or without 75% nitrous oxide inhalation, for 90 min. The brain was fixed with fixative, and brain sections, including the paraventricular nucleus of the hypothalamus, were double immunostained with c-Fos and CRF antibodies to assess the activation of CRF-containing neurons. In other groups of rats, the effect of propofol on nitrous oxide antinociception was evaluated with tail flick latency tests. Both inhaled isoflurane and intravenous propofol inhibited nitrous-oxide-induced activation of CRF neurons, suggesting that these general anesthetics may inhibit one of the analgesic mechanisms of nitrous oxide. Indeed, propofol inhibited the antinociceptive action of nitrous oxide, as evaluated with tail flick latencies (TFL).

Keywords: Nitrous oxide; Corticotropin releasing factor; Isoflurane; Propofol; c-Fos

1. Introduction

Previous studies have indicated that the antinociceptive action of nitrous oxide is mediated by the activation of brainstem noradrenergic neurons and descending inhibitory pathway (Guo et al., 1999; Guo et al., 1996; Sawamura et al., 2000; Zhang et al., 1999). We previously reported that nitrous oxide activates corticotropin releasing factor (CRF)-containing neurons in rat paraventricular nucleus of the hypothalamus (Sawamura et al., 2003). CRF is widely accepted as a key substance mediating physiological responses to various stressors. Nitrous-oxide-induced activation of intracerebral CRF system leads to the activation of brainstem noradrenergic neurons and resultant antinociceptive effect because the intracerebroventricular injection of CRF antagonist blocked these effects of nitrous oxide (Sawamura et al., 2003).

In clinical practice, nitrous oxide is usually administered together with inhaled or intravenous anesthetics. In this study, we evaluated the effect of inhaled isoflurane and intravenous propofol on nitrous-oxide-induced activation of CRF system in rat brain. Immunohistochemical methods were employed, and the expression of c-Fos protein was used as a marker of neuronal activation. Furthermore, as several studies have suggested that nitrous oxide analgesia is inhibited by inhaled anesthetics coadministered with nitrous oxide (Goto et al., 1996; Janiszewski et al., 1999), we examined whether propofol infusion inhibits the antinociceptive effect of nitrous oxide using tail flick latency (TFL) tests in rats.

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2. Materials and methods

2.1. Animals

These experiments were reviewed and approved by Tokyo University Ethical Committee on Animal Research (Tokyo, Japan). Male Sprague–Dawley rats (250–300 g) were housed in a temperature- and humidity-controlled environment and were maintained on a 12-h light/dark cycle. Food and water were available ad libitum. Rats had been habituated to the experimental condition for 2 h for each of five consecutive days.

2.2. Immunohistochemistry

Rats were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and transcardially perfused with 100 ml of 0.1 M sodium phosphate buffer saline, followed by 500 ml of 2% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer cooled to 4 °C. After decapitation, the whole brain was removed and immersed in the same fixative for 24 h at 4 °C. Tissues were then stored overnight in 30% sucrose solution in 0.1 M phosphate buffer at 4 °C for cryoprotection. The brain was sliced into 40-μm-thick sections with a cryotome (CM1800, Leica, Heidelberg, Germany) at −15 °C. Every third section, including the paraventricular nucleus (coordinates: 1.5–2.0 mm caudal to the bregma, 0.5–1.0 mm lateral to the midline and 5.5–6.5 mm below the dura), was retained and placed in the phosphate buffer solution.

Sections were then double-stained for c-Fos and CRF as described previously (Sawamura et al., 2003). As a result, c-Fos positive nuclei were stained black, with cytoplasmic CRF expression appearing brown. Care was taken to process samples from different groups simultaneously to minimize the effect of fluctuation in staining.

2.3. Tail flick latency test

Tail flick latencies (TFL) were determined from the mean of two consecutive latencies using a tail flick apparatus (Muromachi Ikakikai, Tokyo, Japan). The interstimulus interval was approximately 1 min. A high-intensity light was focused on the ventral surface of the middle third of the tail, and the time for the animal to move its tail out of the light beam was automatically recorded. Two different sites of the middle third of the tail were exposed to the light beam to minimize the risk of tissue damage. The same light stimulus intensity was used in all experiments and was preset to give a mean latency of 2.5 s under room air. A cut-off time of 6 s was used to avoid the possibility of tissue damage. If no response had occurred by this time, a value of 6 s was ascribed to the test. Tail skin temperature was measured immediately before the tail flick test with a thermocouple probe mounted on a heat-insulating plate (2×2 cm). For temperature measurement, the probe was kept in contact with the ventral surface of the tail close to the point heated in the tail flick test. The rats were kept on a heating blanket throughout the TFL tests to maintain the tail temperature within 1 °C of 30 °C.

2.4. Effect of isoflurane on nitrous-oxide-induced activation of CRF neurons

Rats were individually exposed for 90 min to isoflurane (0%, 0.6%, 1.0% and 1.5%), with or without 75% N_2O, in a Plexiglas chamber (25×25×30 cm, n=6 for each group). An airway gas monitor (Model 254, Datex, Helsinki, Finland) continuously monitored the concentrations of isoflurane, oxygen, nitrous oxide and carbon dioxide in the chamber, and flow rates were adjusted to maintain the desired concentrations. After 90 min of gas exposure, rats were anesthetized, perfused with fixative, and the brains were removed as described above. Every third brain section, including the paraventricular nucleus, was picked up and double immunostained for c-Fos and CRF expression.

2.5. Effect of propofol on nitrous-oxide-induced activation of CRF neurons

Under brief halothane anesthesia, rats were implanted with a venous cannula in the distal portion of the tail. They were then infused with intravenous propofol (0, 0.1 or 0.2 mg/kg/min) for 20 min. The animals were then exposed for 90 min to 75% N_2O or room air in a Plexiglas chamber, with the propofol infusion continued (n=6 for each group). Rats were sacrificed, and the brains were removed. The brain sections were then double immunostained for c-Fos and CRF expression as described above.

2.6. Effect of propofol on nitrous-oxide-induced antinociception

After a baseline measurement of TFL ($TFL_{baseline}$), rats were implanted with a venous cannula in the distal portion of the tail under brief halothane anesthesia. Animals were then divided into three groups, and propofol infusion was started at 0, 0.1 and 0.2 mg/kg/min, respectively (n=6 for each group). With the propofol infusion continued, TFL measurements were performed 20 min later (TFL_{Pro}) and repeated after 20 min of exposure to 75% nitrous oxide ($TFL_{Pro+N2O}$).

2.7. Statistical analysis

Three brain sections containing the largest number of CRF positive neurons was selected in each rat, and the ratio of c-Fos positive neurons among CRF positive neurons was determined. TFL_{Pro} and $TFL_{Pro+N2O}$ were respectively converted to percent maximal possible effect (%MPE) using

$TFL_{baseline}$ and cut-off time of 6 s. Results were expressed as mean±S.D., and P values less than 0.05 were considered significant. Data were analyzed with an analysis of variance and post hoc comparisons by Dunnett tests.

3. Results

c-Fos expression was hardly observed in rats that were kept under the room air without receiving nitrous oxide, isoflurane or propofol (data not shown).

Fig. 1 demonstrates the c-Fos expression in CRF-containing neurons of rat paraventricular nucleus. Remarkable c-Fos induction was observed in rats exposed for 90 min to 75% nitrous oxide (Panel A). This effect of nitrous oxide was inhibited by the concomitant administration of 1.5% isoflurane (Panel B).

Fig. 2 shows the effect of isoflurane on nitrous-oxide-induced c-Fos expression in the CRF neurons of the paraventricular nucleus. In control rats, the inhalation of 75% nitrous oxide induced c-Fos in 86±17% of CRF neurons. Isoflurane dose dependently suppressed the c-Fos positive ratio of CRF neurons (84±16%, 25±15% and 8±12% for 0.6%, 1.0% and 1.5% isoflurane, respectively; P<0.0001). Exposure to isoflurane alone (0.6%, 1.0% and 1.5%) did not induce c-Fos in the CRF neurons (data not shown). No significant difference was found in the number of CRF-containing neurons among groups.

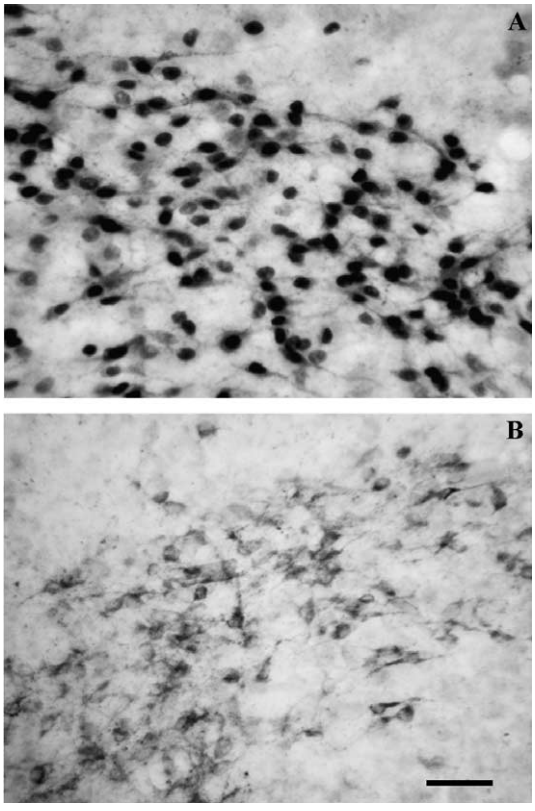

Fig. 1. c-Fos induction in corticotropin releasing factor (CRF)-containing neurons of rat paraventricular nucleus of hypothalamus. CRF positive cytoplasm and c-Fos positive nuclei look gray and black, respectively, in this black-and-white photograph. Remarkable c-Fos induction is observed after 90 min of exposure to 75% nitrous oxide (A), while c-Fos expression is rare after 90 min of exposure to 75% nitrous oxide plus 1.5% isoflurane (B). Bar=20 μm.

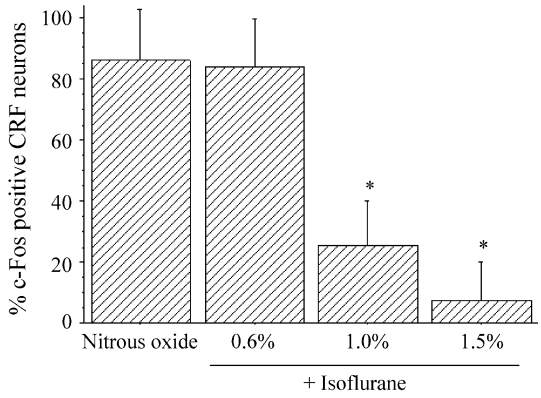

Fig. 2. Effect of isoflurane on nitrous-oxide-induced c-Fos expression in the CRF positive neurons of paraventricular nucleus. The percentage of c-Fos positive neurons among CRF positive neurons was dose dependently reduced by the concomitant administration of isoflurane. *P<0.0001 vs. nitrous oxide; n=6 for each group.

Fig. 3 shows the effect of propofol infusion on nitrous-oxide-induced c-Fos expression in the CRF neurons of the paraventricular nucleus. Propofol dose dependently suppressed the c-Fos positive ratio of CRF neurons (86±18%, 78±17% and 17±15%, for 0, 0.1 and 0.2 mg/kg/min propofol, respectively; P<0.0001). Propofol infusion alone (0.1 and 0.2 mg/kg/min) did not induce c-Fos in the CRF neurons (data not shown). No significant difference was found in the number of CRF-containing neurons among groups.

Our preliminary experiments showed that cannulation of the tail vein had no effect on the baseline tail flick latency. The effects of propofol infusion on the antinociceptive action of nitrous oxide are demonstrated in Fig. 4. There was no significant difference in TFL_{Pro} among the three

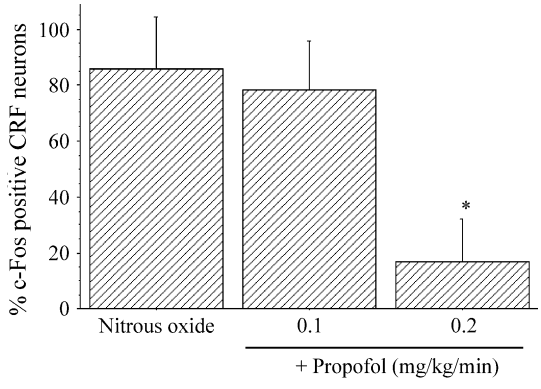

Fig. 3. Effect of propofol infusion on nitrous-oxide-induced c-Fos expression in the CRF positive neurons of paraventricular nucleus. The percentage of c-Fos positive neurons among CRF neurons was dose dependently reduced by th concomitant administration of propofol. *P<0.0001 vs. nitrous oxide; n=6 for each group.

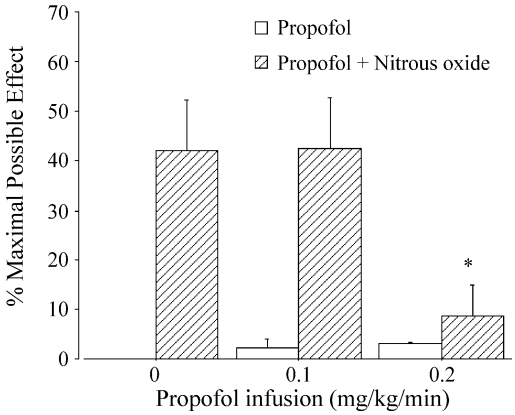

Fig. 4. Effect of propofol on the antinociceptive action of nitrous oxide as evaluated with tail flick latency tests. Tests were performed under propofol infusion alone (0, 0.1 and 0.2 mg/kg/min) and repeated after 20 min of exposure to nitrous oxide with the propofol infusion continued. Results were converted to percent maximum possible effect using control latency and cut-off time (6 s). *P<0.05 vs. zero propofol infusion; n=6 for each group.

propofol doses (0, 0.1 and 0.2 mg/kg/min), indicating that these doses of propofol exert no antinociceptive action. $TFL_{Pro+N2O}$ was significantly decreased at the propofol infusion of 0.2 mg/kg/min (9±14% MPE) as compared with zero propofol infusion (42±27% MPE, P<0.05), indicating that propofol inhibited the antinociceptive action of nitrous oxide in rats.

4. Discussions

Our results indicated that nitrous-oxide-induced activation of CRF neurons in rat paraventricular nucleus was dose dependently inhibited by concomitant administration of isoflurane or propofol. The activation of CRF system constitutes, at least, part of the analgesic mechanisms of nitrous oxide. We previously reported that inhalation of nitrous oxide induces the activation of CRF neurons, as evidenced by enhanced c-Fos expression in these neurons (Sawamura et al., 2003). This is not necessarily a direct evidence of CRF release by nitrous oxide. However, the stimulation of CRF system by nitrous oxide may lead to the activation of noradrenergic neurons in the locus coeruleus and antinociceptive action mediated by descending inhibitory pathway because the intracerebroventricular administration of CRF antagonist blocked these effects of nitrous oxide in rats (Sawamura et al., 2003).

Isoflurane and propofol are frequently administered together with nitrous oxide in clinical practice of anesthesia. Our results in immunohistochemical study may suggest that these general anesthetics can possibly inhibit the analgesic mechanism of nitrous oxide mediated through the CRF system and descending inhibition. In fact, Goto et al. (1996) demonstrated that both halothane and isoflurane dose dependently antagonized nitrous oxide analgesia in rats. Janiszewski et al. (1999) also reported that subanesthetic concentration of sevoflurane inhibited nitrous oxide analgesia in healthy volunteers. Our behavioral study also indicated that propofol (0.2 mg/kg/min) inhibited the antinociceptive action of nitrous oxide in rats, as evaluated with the tail flick latencies. This is compatible with our immunohistochemical results because the same dose of propofol inhibited nitrous-oxide-induced activation of CRF neurons. It is, however, possible that intracerebral regions other than CRF neurons are stimulated by nitrous oxide inhalation and are involved in its analgesic action. General anesthetics may suppress these regions and inhibit nitrous oxide analgesia. On the other hand, an additive interaction of propofol and nitrous oxide has been reported in hypertensive responses to nerve stimulation in rabbits (Ichinohe et al., 2000). These contradictory results suggest that multifarious actions of nitrous oxide may exist, some of which are inhibited and the others are enhanced by concomitant use of general anesthetics.

It may be speculated that nitrous-oxide-induced neuronal activation was caused by "inadequate anesthesia" and was inhibited by "deepening" anesthesia with additional isoflurane and propofol. However, because neither low concentration of isoflurane (0.6 and 1%) nor low dose propofol (0.1 mg/kg/min) induced c-Fos by themselves, and adding nitrous oxide to these anesthetics led to c-Fos induction in CRF neurons, CRF neuronal activation can be considered as a specific effect of nitrous oxide rather than results of inadequate anesthesia.

In summary, we demonstrated that both isoflurane and propofol inhibit the nitrous-oxide-induced activation of CRF neurons in rats. Because part of nitrous oxide analgesia is mediated through the CRF system, our results suggest that these general anesthetics can inhibit one of the analgesic mechanisms of nitrous oxide. Indeed, our behavioral study in rats indicated that propofol infusion inhibited the antinociceptive action of nitrous oxide, as evaluated with tail flick latency tests.

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