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Involvement of leukotriene pathway in the development of sevoflurane-induced pica in rats

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Abstract

We previously reported that sevoflurane-induced pica, kaolin ingestion behavior, in rats has the potential to reflect post-operative nausea and vomiting (PONV) in humans. It is well-known that corticosteroids, which inhibit both prostaglandin and leukotriene syntheses due to phospholipase A2 inhibition, are effective for reducing PONV; however, the precise mechanisms remain unclear. We investigated the involvement of the prostaglandin or leukotriene pathway in the development of sevoflurane-induced pica. We found that sevoflurane-induced pica was effectively inhibited by pretreatment with a leukotriene receptor antagonist (montelukast) or an inhibitor of 5-lipoxygenase (zileuton), rather than an inhibitor of cyclooxygenase (flurbiprofen). Furthermore, we observed that sevoflurane significantly increased urinary leukotriene excretion and 5-lipoxygenase mRNA expression in the spleen, but not hypothalamus. These results suggest that the production of leukotriene may lead to the development of sevoflurane-induced pica in rats, and that inhibition of the leukotriene pathway could be potentially useful for the treatment of PONV.

Keywords

leukotriene; 5-lipoxygenase; pica; postoperative nausea and vomiting;

Introduction

Postoperative nausea and vomiting (PONV) occur in 30% of surgical patients under general anesthesia (Gan, 2006). Serotonin 5-HT₃, neurokinin NK₁ receptor antagonists, and corticosteroids are used to reduce the incidence and severity of PONV (Horn et al. 2014). Recent studies reported that dexamethasone combined with other antiemetic agents provided better prophylaxis than single antiemetic agents against postoperative nausea and vomiting (Som et al. 2016). The main pharmacological effect of glucocorticoids including dexamethasone is the inhibition of both prostaglandin and leukotriene syntheses due to phospholipase A2 inhibition (De Castro et al. 1995). From these findings, it has been postulated that the etiology of PONV is involved in the inflammatory mediators and pathways; however, the precise mechanisms remain unclear. We previously reported that sevoflurane-induced pica, a craving to eat non-food materials such as kaolin, in young female rats can be used to assess PONV in humans (Yamamoto et al. 2015). Therefore, in this study, we examined the effects of the prostaglandin or leukotriene pathway on the development of sevoflurane-induced pica in rats, and investigated the mRNA expression of 5-lipoxygenase (5-LOX), a key enzyme in leukotriene biosynthesis, in the medulla oblongata of sevoflurane-treated mice.

Methods

All experimental protocols were approved in accordance with the Animal Experimental Committee of Osaka University (name of committee: The Animal Care Committee of the School of Allied Health Sciences, Faculty of Medicine, Osaka University, the approval number: No. 26-05-02), and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council,

2011). We previously found that female rats at the age of 8 weeks were more susceptible to the induction of sevoflurane-induced pica than male, females at the age of 20 weeks, and ovariectomized females (Yamamoto et al. 2015). Naive specific-pathogen-free (SPF) female (8 weeks old, body weight: 180-210 g) Wistar/ST rats were obtained from Japan SLC (Shizuoka). They were housed in individual home cages (25 x 20 x 20 cm) that were placed in a room under SPF conditions, with a regular light/dark cycle (lights on 6:00–18:00) and at a constant temperature (approximately 24°C) and humidity (approximately 50%). During habituation and the experimental period, rats were allowed free access to water, standard commercially available chow (CE-2, CLEA Japan, Inc., Tokyo, Japan), and handmade kaolin pellets prepared according to a previously reported method (Yamamoto et al. 2015, 2016). The food and kaolin pellets were provided in their respective stainless steel containers (5 x 5 x 10 cm) placed in the home cage. After more than one week of habituation, the rats were administered sevoflurane (Pfizer Japan Inc., Tokyo, Japan). The anesthetic method was as follows. Rats were placed in a sealed acrylic box (40 x 40 x 30 cm) and exposed to 3% sevoflurane using a vaporizer (BS-400T; Brain Science Idea Co., Ltd., Osaka) for induction. After the confirmation of immobilization, rats were removed from the acrylic box and continuously exposed to 1.5% sevoflurane with a rodent face mask. The concentration of sevoflurane was determined by confirmation of the loss of the righting reflex in each rat. To maintain a core temperature of around 37°C, all rats were placed on a heating pad during anesthesia, and the body temperature was monitored using a rectal probe. The 6 hour duration of sevoflurane anesthesia was selected according to a previously reported method (Yamamoto et al. 2016). The time when the sevoflurane inhalation anesthesia was stopped was set in accordance with the start time of the dark phase (at 18:00). All rats were randomized into seven groups after returning to their home cages, and were simultaneously injected with

one of the following drugs: a leukotriene receptor antagonist, montelukast (Singulair[®], 2 (n = 8) or 5 (n = 7) mg/kg, intragastrically (i.g.), MSD K.K., Tokyo, Japan), an inhibitor of 5-LOX, zileuton (2 (n = 5) or 5 (n = 5) mg/kg, i.g., Sigma-Aldrich, St. Louis, MO, USA), or an inhibitor of cyclooxygenase, flurbiprofen (Ropion[®] inj, 5 (n = 5) or 10 (n = 5) mg/kg, (i.g.), Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) at the end of sevoflurane anesthesia. Control animals received an i.g. injection of saline (1 mL/100 g body weight, n = 6). Their kaolin intakes were measured on the day after receiving the anesthetic agent according to our reported method (Yamamoto et al. 2016). There were five to eight rats in each of the experimental groups. The experimental schedule is shown in Figure 1 a. In this study, the estrus cycles of female rats were not considered throughout the experiment. All drugs were purchased from Katayama Chemical Industries (Osaka, Japan). Montelukast and zileuton were suspended in an aqueous suspension of 0.5% carboxymethylcellulose (Sigma-Aldrich Japan). Flurbiprofen was diluted with physiological saline. Doses are expressed as the free base. Since animals were not used more than once, they were sacrificed by an i.p. injection of excess sodium pentobarbital (100 mg/kg) at the end of the experiment.

In another series of experiments, rats were administered sevoflurane anesthesia for 6 hours and then their hypothalamus and spleen were removed at 6 hours after finishing the sevoflurane administration. For the sham control experiment, rats were placed in the box without anesthesia for 6 hours. Total RNA in the hypothalamus or spleen was extracted using the Total RNA Extraction System (Viogene, New Taipei, Taiwan), according to the manufacturer's instructions. RNA was converted into first-stranded cDNA with the reverse transcriptase enzyme kit (ReverTra Ace[®], TOYOBO Life Science, Osaka, Japan), which was then used as a template for the reverse transcription polymerase chain reaction (RT-PCR) with 5-LOX and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific primers. The

sequences of primers and thermalcycler (GeneAtras G02, Astec, Co., LTD. Fukuoka, Japan) conditions for RT-PCR are listed in Table 1. PCR products were separated on 3% agarose gels (Nippon GENE, Tokyo, Japan) by electrophoresis and stained with a 1/10,000 dilution of GelGreen TM Nucleic AcidGel Stain (Biotium, Hayward, CA, USA). Gels were captured with E-graph (AE-900, ATTO, Tokyo, Japan) and their band densities were analyzed for quantification using the ATTO CS Analyzer ver.3.0. The relative level of 5-LOX mRNA was calculated with the respective average expression value of the control sample which was arbitrarily set as 1.0. There were six rats in each of the experimental groups. The assay was performed with two biological replicates, each with two technical replicates. The experimental schedule is shown in Figure 2 a.

Finally, since cysteinyl leukotrienes are known to be excreted in urine as intact leukotriene E4 and its metabolites, six-hourly urine samples were collected between 6 and 12 hours after sevoflurane administration. All cysteinyl leukotriene concentrations in the urine samples were analyzed using an enzyme immunoassay kit (Item Number 500390, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. For the control experiment, rats were placed in the box without anesthesia for 6 hours. There were five rats in each of the experimental groups. The assays was performed with two biological replicates, each with two technical replicates.

The data are expressed as the mean value $\pm$ standard deviation. All differences were analyzed using the Mann-Whitney U test for the comparison of two groups, and Kruskal-Wallis test followed by post-hoc Dunn's test for multiple comparison where appropriate. A P-value of less than 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). The primary outcome of this study was the effects of the cysteinyl

leukotriene ligands on the amounts of kaolin intake in rats treated with sevoflurane. The secondary outcomes were the expression of 5-LOX mRNA in the hypothalamus and spleen, and urinary excretion of leukotriene levels. A power analysis and sample size calculation were performed using JMP 14 (SAS Institute Inc., Cary, NC, USA). In the experiment of sevoflurane-induced pica in rats, when an alpha-error was set at 0.05, total sample size was 35, number of groups was 7, and then the power was evaluated as 97.6%; therefore, we considered that the number of animals in each group should be at least 5.

Results

During the habituation period, all rats ate between 10 and 15 g of food pellets and less than 0.4 g of kaolin pellets. Rats treated with saline showed a significantly increased kaolin intake after exposure to sevoflurane ($n = 6$: before experiment: 0.11 ± 0.15 g; after experiment: 2.12 ± 1.18 g, $p < 0.01$), but both intakes returned to normal levels 2 days after the experiment. None of rats exhibited any abnormality other than pica behavior throughout the observation period. None of the treatment drugs used induced any abnormality in rats (data not shown). As shown in Figure 1 b, pretreatment with higher doses of montelukast and zileuton significantly inhibited sevoflurane-induced pica. The inhibitory effects of zileuton were superior to those of montelukast; however, neither dose of flurbiprofen had an effect on sevoflurane-induced pica. A six-hour duration of sevoflurane anesthesia led to significantly higher 5-LOX mRNA expression levels in the spleen but not hypothalamus (Figure 2 b). The sevoflurane administration caused a significant increase in urinary excretion of leukotriene levels compared with control rats (control ($n = 5$): 819.6 ± 86.1 pg/mL; sevoflurane ($n = 5$): 936.9 ± 15.8 pg/mL, $p < 0.05$).

Discussion

PONV is not a life-threatening symptom, but it is distressing for patients who have undergone surgery. A previous study reported that a single dose of dexamethasone reduces both the incidence of PONV and the need for rescue anti-emetic drugs (Som et al. 2016). A well-known medicinal effect of dexamethasone is the prevention of arachidonic acid release and synthesis of inflammatory mediators such as prostaglandin, leukotriene, and thromboxane by inhibiting the activity of phospholipase A₂ (De Castro et al. 1995). Chebolu et al. reported that cysteinyl leukotrienes such as leukotriene C₄ (LTC₄), D₄ (LTD₄), and E₄ (LTE₄) induced vomiting in the least shrew, and pranlukast, which is a cysteinyl leukotriene receptor-1 antagonist, dose-dependently and significantly inhibited LTC₄-induced vomiting (Chebolu et al. 2010). More recently, Darmani et al. reported that pranlukast suppressed the cisplatin-induced vomiting that occurs more than 24 hours after the administration of cisplatin in the least shrew (Darmani et al. 2017). These findings suggest a leukotriene CysLT₁ receptor-mediated emetogenic effect. It is considered that volatile anesthetic agents have antioxidant and anti-inflammatory properties (Cruz et al. 2017; Blum and Zuo 2013); however, Takala et al. (2004) reported significant increases in LTC₄ after a six-hour duration of sevoflurane anesthesia in the pig, suggesting that the long-term duration of sevoflurane anesthesia may influence the induction of 5-LOX activation. Furthermore, Okuno et al. (2017) reported that propofol, which has an effective antiemetic property (Soppitt et al. 2000), attenuated the production of 5-LOX-related arachidonic acid derivatives including LTC₄ and LTD₄. Leukotriene is known to induce a release of substance P (Bloomquist and Kream 1990). The increased release of substance P induced vagal afferent activity via adjacent NK₁ receptors, and activated the central emetic circuitry in the medulla oblongata. Previous studies demonstrated that an NK₁ antagonist inhibited sevoflurane-induced vomiting

in humans and *suncus murinus* (Horn et al. 2014; Gardner and Perren 1998). Based on our observations that a six-hour duration of sevoflurane anesthesia significantly increased the expression of 5-LOX mRNA, the urinary excretion of cysteinyl leukotrienes, and pretreatment with montelukast and zileuton significantly inhibited sevoflurane-induced pica in rats, the sevoflurane-induced activation of 5-LOX and subsequent production of leukotriene contribute to the development of anesthetic-induced pica in rats. Therefore, the administration of NK₁ receptors antagonists combined with LOX inhibitors or a leukotriene receptor antagonist led to further synergistic effects for the prevention of PONV in human patients. Further experiments are needed to elucidate the role of leukotrienes using this animal model by confirming whether LOX inhibitors modify the sevoflurane-induced changes in the expression of 5-LOX protein. Since we have reported that pica in rats is useful to evaluate nausea and vomiting in humans (Yamamoto et al. 2011), we consider that the results of this study suggest the clinical benefit of the therapeutic effect of CysLT₁ receptor antagonist against PONV. However, according to clinical studies (Altman et al. 1998; Israel et al. 1993), the daily clinical doses of montelukast and zileuton are calculated as about 0.16 and 40 mg/kg per human unit weight, respectively, and the doses used in this study are different from these doses; therefore, we need to recognize these limitations of this study, and conduct further experiments in order to identify the most suitable dose and injection times of CysLT₁ receptor antagonist for the prevention of PONV.

Conclusion

In summary, leukotriene production in the medulla was involved in the development of sevoflurane-induced pica in rats.

Abbreviations list

5-LOX: 5-lipoxygenase

CysLT₁: cysteinyl leukotriene receptor-1

LTC₄: leukotriene C₄

LTD₄: leukotriene D₄

LTE₄: leukotriene E₄

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

PONV: postoperative nausea and vomiting

RT-PCR: reverse transcription polymerase chain reaction

SPF: specific-pathogen-free

Draft

Conflict of Interest

None of the authors have competing interests to declare.

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Figure legends

Figure 1: The effects of a leukotriene receptor antagonist (montelukast: 2 or 5 mg/kg), an inhibitor of 5-lipoxygenase (zileuton: 2 or 5 mg/kg), and an inhibitor of cyclooxygenase (flurbiprofen: 5 or 10 mg/kg) on kaolin intakes in rats treated with sevoflurane. (a) Experimental schedule. (b) Columns and bars represent the mean $\pm$ standard deviation of cumulative kaolin intakes measured 24 h after sevoflurane administration. The number of animals in each group is indicated in parentheses. Each agent or saline was intragastrically administered after finishing the inhalation of sevoflurane (1.5%, 6 h). The data were analyzed for significant differences using the Kruskal-Wallis test followed by post-hoc Dunn's test for multiple comparisons. * $P < 0.05$ and ** $P < 0.01$ vs. control group.

Figure 2: The effects of sevoflurane on the expression of 5-lipoxygenase (5-LOX) mRNA in the hypothalamus and spleen of rats and on the urinary excretion of leukotriene. (a) Experimental schedule for 5-LOX mRNA expression. (b) Columns and bars represent the mean $\pm$ standard deviation, respectively, expressed as a ratio of 5-LOX to glyceraldehyde-3-phosphate dehydrogenase. The number of animals in each group is indicated in parentheses. The relative level of 5-LOX mRNA was calculated with the respective average expression value of the control sample, which was arbitrarily set as 1.0. The number of animals in each group is indicated in parentheses. The data were analyzed for significant differences using the non-parametric Mann-Whitney U test. * $P < 0.05$ vs. each respective control group.

(a)

(b)

Figure 1

(a)

(b)

Figure 2

Table 1: List of primer sequences and thermal conditions used for RT-PCR analysis in this study.

Primer	Sequence (5'-3')	PCR product size	PCR conditions
5- LOX	sense TCACCATTGCCATCAACACT	241 bp	94 °C for 3 min, 33 cycles
	antisense TGGATAGCTTCCCACACGA		94 °C for 20 sec 62 °C for 20 sec 72 °C for 40 sec 72 °C for 5 min
GAPDH	sense ATGGGAAGCTGGTCATCAAC	484 bp	94 °C for 3 min, 28 cycles
	antisense TTCAGCTCTGGGATGACCTT		94 °C for 20 sec 62 °C for 20 sec 72 °C for 40 sec 72 °C for 5 min