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Activation of the canonical nuclear factor-κB pathway is involved in isoflurane-induced hippocampal interleukin-1β elevation and the resultant cognitive deficits in aged rats

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ABSTRACT

Although much recent evidence has demonstrated that neuroinflammation contributes to volatile anesthetic-induced cognitive deficits, there are few existing mechanistic explanations for this inflammatory process. This study was conducted to investigate the effects of the volatile anesthetic isoflurane on canonical nuclear factor (NF)-κB signaling, and to explore its association with hippocampal interleukin (IL)-1β levels and anesthetic-related cognitive changes in aged rats. After a 4-h exposure to 1.5% isoflurane in 20-month-old rats, increases in IκB kinase and IκB phosphorylation, as well as a reduction in the NF-κB inhibitory protein (IκBα), were observed in the hippocampi of isoflurane-exposed rats compared with control rats. These events were accompanied by an increase in NF-κB p65 nuclear translocation at 6 h after isoflurane exposure and hippocampal IL-1β elevation from 1 to 6 h after isoflurane exposure. Nevertheless, no significant neuroglia activation was observed. Pharmacological inhibition of NF-κB activation by pyrrolidine dithiocarbamate markedly suppressed the IL-1β increase and NF-κB signaling, and also mitigated the severity of cognitive deficits in the Morris water maze task. Overall, our results demonstrate that isoflurane-induced cognitive deficits may stem from upregulation of hippocampal IL-1β, partially via activation of the canonical NF-κB pathway, in aged rats.

1. Introduction

Postoperative cognitive dysfunction (POCD) in the elderly has emerged as a major health concern [1]. It is associated with premature departure from the workforce, increased disability, and early mortality [2]. Unfortunately, the pathophysiology of POCD remains elusive.

The potential risk factors for POCD can be classified into three categories from the patient, the surgery or the anesthesia [1]. Many recent animal studies [3–6] and clinical observations [7,8] have supplied evidence that anesthetics, particularly inhalational anesthetics, may play a role in cognitive deficits. Isoflurane, an inhalation anesthetic that is widely used clinically, has been shown to induce POCD through cytokine-dependent neuroinflammatory mechanisms, in which isoflurane increases the production of interleukin 1 (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in a rat or mouse brain [3,9]. Nevertheless, the exact signaling mechanisms by which isoflurane mediates increases in these proinflammatory cytokines remain to be elucidated.

Nuclear factor (NF)-κB is one of the most critical transcription factors involved in inflammation [10]. The NF-κB family comprises of RelA/p65, RelB, c-Rel, p50, and p52, RelA/p50 heterodimer being the most abundant and widely expressed [11]. Multiple proinflammatory signals activate NF-κB, mostly through inhibitor of NF-κB protein (IκB) kinase (IKK)-dependent phosphorylation of IκB, leading to IκB ubiquitination and degradation. This ultimately leads to nuclear translocation of NF-κB and induction of transcription of the target genes [11], including the proinflammatory cytokine IL-1β [10]. NF-κB signaling can be regulated by IκBs and IKKs through various routes. The most frequently observed is the canonical path-

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way, which is characterized by phosphorylation of IκBα on serine residues 32 and 36 and proteasome degradation, and nuclear translocation of RelA/p65 depending on the catalytic subunits IKKα/β [12].

Recent *in vivo* and *in vitro* studies have revealed that NF-κB activation may be involved in mediating the neuroinflammation in models of Alzheimer's disease [13,14], which has a similar molecular pathological mechanism to POCD. Therefore, the present study aimed to determine whether NF-κB signaling is involved in isoflurane-induced neuroinflammation and cognitive impairment *in vivo*. Specifically, we hypothesized that isoflurane would induce neuroinflammation through activation of the canonical NF-κB pathway in aged rats.

2. Materials and methods

2.1. Animals

Aged male Sprague–Dawley rats (20 months of age; weight: 550–600 g) were used for all experiments. They were bred and maintained under standardized housing conditions with food and water *ad libitum*. The experimental protocol was approved by the Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (Approval No. LA2012-38).

2.2. Experiment protocols

2.2.1. Experiment A

To study the effects of isoflurane exposure on the NF-κB signaling pathway activity, rats were randomly assigned to isoflurane ($n = 24$) or control ($n = 8$) groups and exposed to isoflurane or vehicle gas, respectively. The expression levels of hippocampal IL-1β and two glial cell activation markers, cluster of differentiation 11b (CD11b) and glial fibrillary acidic protein (GFAP), were dynamically examined at 1, 3, 6, 12, and 24 h after isoflurane exposure using enzyme-linked immunosorbent assay (ELISA) and Western blotting ($n = 4$ per time point), respectively. Meanwhile, the activation of NF-κB, including IκBα degradation and phosphorylation of IκBα and IKKα/β, was also assessed by Western blotting. NF-κB p65 nuclear translocation was observed at 6 h after anesthesia by immunofluorescence ($n = 4$ each).

2.2.2. Experiment B

To evaluate the role of the NF-κB signaling pathway in isoflurane exposure, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (Sigma–Aldrich, St. Louis, MO) was used for blocking studies. Rats were randomly assigned to control, ISO, PDTC + ISO, and PDTC groups ($n = 13$ per group). The rats in the PDTC + ISO and PDTC groups were both intraperitoneally administered with PDTC at 100 mg/kg in saline (total volume: 0.5 ml) 1 h before exposure to isoflurane or vehicle gas, respectively. The rats in the other two groups received an identical volume of saline. This dosing protocol of PDTC has been shown to effectively inhibit lipopolysaccharide-induced IκBα degradation and the resultant NF-κB activation in rats [15]. Subsequently, the rats in the ISO and PDTC + ISO groups received isoflurane exposure, while the rats in the other two groups were exposed to vehicle gas without anesthetic for an equivalent period of time.

At 6 h after anesthesia, four rats per group were euthanized for hippocampal harvesting. Half of each hippocampus was used for Western blotting analyses of the phosphorylated IKK (p-IKK), p-IκBα, and IκBα levels, and the other half was used for ELISA detection of the IL-1β levels ($n = 4$). Hippocampal-dependent spatial memory ability was evaluated using the Morris water maze (MWM) test ($n = 9$ per group).

2.3. Isoflurane exposure

The protocol for isoflurane exposure was based on our previous studies [16,17]. Briefly, rats were placed in a temperature-controlled, transparent anesthetic chamber. During exposure, the chamber was gassed with 1.5% isoflurane (Baxter Healthcare, Deerfield, IL) through a calibrated isoflurane vaporizer, carried by 100% oxygen for 4 h. Standard soda lime was placed at the bottom of the container to clear the carbon dioxide. The concentrations of isoflurane, oxygen, and carbon dioxide in the chamber were continuously analyzed with a gas monitor (Datex-Ohmeda, Louisville, CO). After anesthesia, the rats received 100% oxygen until they regained consciousness.

2.4. Blood gas analysis

To determine whether isoflurane anesthesia caused physiologic side effects such as hypoxia, hypercapnia, or hypoglycemia, five rats in the various treatment groups were selected as cardiorespiratory control animals (total: $n = 20$). Two milliliters of blood was immediately drawn by cardiac puncture at the end of the isoflurane exposure. Arterial blood gases (ABG) and blood glucose measurements were performed using a portable blood gas analyzer (OPTI Medical Systems, Roswell, GA) and an One Touch Ultra blood glucose monitoring system (Life Scan Inc., Milpitas, CA), respectively. The cardiorespiratory control rats were not used for any other part of the study.

2.5. ELISA

The homogenates from the hippocampus were centrifuged at 10,000×*g* for 10 min at 4 °C as described previously [14]. The concentration of IL-1β in the supernatant fluid was measured using an ELISA kit (Rapidbio Lab, West Hills, CA), according to the manufacturer's instructions. Each experimental condition was tested in three different wells and measured in duplicate.

2.6. Western blotting

Western blot analyses were performed to determine the expression levels of CD11, GFAP, p-IKKα/β, p-IκBα, and IκBα as previously described [18], with the following modifications. 60 μg protein per lane was separated by 10% SDS–PAGE. After transfer to membranes, the proteins were probed with the following primary antibodies: anti-CD11b (1:500; Millipore, Billerica, MA); anti-GFAP and anti-p-IκBα (1:1000; CST, Danvers, MA); anti-IκBα and anti-pIKK (1:1000; CST). Fluorescently labeled secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE) were used to detect the binding of the primary antibodies. The bound proteins were visualized by scanning the membranes in an Odyssey Infrared Imaging System (LI-COR Biosciences). The results for rats under the different experimental conditions were normalized by the mean values of the corresponding control animals.

2.7. Morphology

Tissue preparation and immunofluorescence staining of brain sections were performed as previously described [17] using an anti-NF-κB p65 primary antibody (1:50; CST) and a fluorescein isothiocyanate-labeled secondary antibody (1:200; Abcam, Cambridge, UK). The nuclei were counterstained with 4,6-diamidino-2-phenyl-indole (1:5000; Roche, Mannheim, Germany).