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Research report

Effect of melatonin on attenuating the isoflurane-induced oxidative damage is related to PKCα/Nrf2 signaling pathway in developing rats

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ABSTRACT

Isoflurane, an inhalational anesthesia, has frequently been used in pediatric anesthesia. However, research indicates that isoflurane can induce oxidative stress and affect neural and cognitive development. Melatonin, an endogenous hormone that exhibits antioxidant functions, can play a neuroprotective role by activating the PKCα/Nrf2 signaling pathway in response to oxidative stress. This study aims to determine whether the effect of melatonin on isoflurane-induced oxidative stress is related to activation of the PKCα/Nrf2 signaling pathway. Rat pups at postnatal day 7 were treated with control or 1.5% isoflurane for 4 h after pretreatment for 15 min with either melatonin (10 mg/kg i.p.) or 1% ethanol. The hematoxylin and eosin staining and transmission electron microscopic examination were used for observation of histopathology. The oxidative stress-related indicators were detected by using assay kits. The western blotting, immunohistochemistry and immunofluorescence were used to detect the activation of PKCα/Nrf2 signaling pathway. Results showed that isoflurane induced nerve damage in the hippocampus, and melatonin could reduce this injury. Oxidative stress-related indicators suggested that isoflurane can significantly increase reactive oxygen species and malondialdehyde levels, and decrease superoxide dismutase and glutathione activity compared with the control group, whereas melatonin ameliorated these indices. Expression of proteins associated with the PKCα/Nrf2 signaling pathway indicated that the neuroprotective effect of melatonin is related to activation of the PKCα/Nrf2 signaling pathway. These results suggest that the attenuating effect of melatonin on isoflurane-induced oxidative stress is related to activation of the PKCα/Nrf2 signaling pathway. These findings promote further research into underlying mechanisms and effective treatments to attenuate anesthesia neurotoxicity.

1. Introduction

For delivery or surgical operation, millions of parturients and neonates undergo procedures involving anesthesia (Lemkuil et al., 2011; Li et al., 2017b; Stratmann et al., 2010). Isoflurane, one of the most common inhalation anesthetics because of its safety, fast onset, rapid recovery and other advantages, has been used in pediatric anesthesia for more than 30 years (Guo et al., 2017). Recently, studies have reported that children exposed to general anesthesia (GA) before 4 years of age have greater risk of learning and memory impairments (Flick et al., 2011; Wilder et al., 2009). Research on neonatal and nonhuman primates also showed that early exposure to GA impaired brain development, for example by inhibiting neurogenesis, and caused long-term neurocognitive impairment (Creeley et al., 2013; Ramage et al., 2013). The hippocampus is a crucial tissue for learning and memory and is particularly vulnerable to anesthesia in a model of isoflurane-induced cognitive deficits in aging mice (Li et al., 2014; Zhang et al., 2012a). Previous studies have demonstrated that isoflurane can damage the hippocampus and subsequently induce hippocampus-dependent learning and memory deficits (Kang et al., 2017).

Mechanisms of isoflurane-induced hippocampus-dependent cognitive deficits are multifactorial. Neuropathogenesis is related with caspase activation, apoptosis, Aβ oligomerization, neuroinflammation, and mitochondrial dysfunction (Bianchi et al., 2008; Brambrink et al., 2010; Wu et al., 2012; Xie et al., 2008; Zhang et al., 2010). Recent findings suggest that oxidative stress is central to GA-induced cognitive deficits (Lee et al., 2015; Li et al., 2017a). However, there remains a lack of clinically effective neuroprotective agents to protect against oxidative

Abbreviations: MLT, melatonin; GSH, glutathione; SOD, superoxide dismutase; ROS, reactive oxygen species; GA, general anesthesia; Nrf2, nuclear factor erythroid 2-related factor 2; Keap 1, kelch-like EHC-associated protein 1; ARE, antioxidant response element; PKC, protein kinase C; HO-1, heme oxygenase-1

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stress induced by isoflurane.

Studies have reported that some Traditional Chinese Medicines, such as resveratrol and compounds like elamipretide, play a neuroprotective role in a model of isoflurane-induced nerve injury. However, these types of drugs have their own limitations. For example, Traditional Chinese Medicines have slow onset. Melatonin, a kind of health-protective remedy, can inhibit oxidative stress via stimulation of antioxidant enzymes (Fischer et al., 2013; Zhang and Zhang, 2014). Melatonin is widely used in experimental studies (Li et al., 2018) and has been shown to reduce early brain injury and neurological symptoms (Ersahin et al., 2009). (Negi et al. (2011)) reported that melatonin modulates neuroinflammation in a model of experimental diabetic neuropathy by decreasing oxidative stress via increased nuclear factor erythroid 2-related factor 2 (Nrf2) expression. Kleszczyński et al. (Kleszczynski et al., 2016) demonstrated that melatonin counteracts ultraviolet radiation-induced injury by enhancing gene expression of phase 2 antioxidative enzymes including heme oxygenase-1 (HO-1). However, the efficacy of melatonin has yet to be studied in the context of oxidative stress induced by isoflurane.

PKC is involved in numerous pathological processes such as oxidative stress and fibrosis (Xu et al., 2010). Members of the PKC family comprise at least 11 isoforms (Huang et al., 2000). PKCα, a classical PKC subtype, plays an important role in antioxidative stress (Chueakula et al., 2018). Studies reported that direct phosphorylation of Nrf2 by PKC is a critical event for the nuclear translocation of Nrf2 in response to oxidative stress (Huang et al., 2000). In addition, the previous study showed that a PKCα inhibitor can reduce Nrf2 expression, which led to HO-1 downregulation (Yun et al., 2010). Nrf2 plays an important role in regulating antioxidant production to maintain cellular redox homeostasis (Kensler et al., 2007; Kong et al., 2011, 2010). Normally, Nrf2 binds to kelch-like EHC-associated protein 1 (Keap1) in the cytoplasm (Taguchi et al., 2011; Vriend and Reiter, 2015). Keap1 is an adaptor subunit of E3 ubiquitin ligase that is an important factor for proteasome-dependent degradation (Jiang et al., 2018). However, under conditions of oxidative stress, the interaction between Keap1 and Nrf2 is disrupted, which activates Nrf2 for translocation into the nucleus, where it binds to antioxidant response elements (ARE). Afterwards, Nrf2 promotes transcription of relevant cytoprotective and antioxidant genes such as HO-1 (Xu et al., 2017; Yadav et al., 2017). Another study suggested that baicalein protects against neurotoxicity by activating PKCα/Nrf2 signaling pathway (Zhang et al., 2012b). Yet, whether the antioxidative effect of melatonin in isoflurane anesthesia is related to the PKCα/Nrf2 signaling pathway has not been thoroughly studied.

We hypothesized that the neuroprotective effect of melatonin alleviates isoflurane-induced oxidative stress by modulating the PKCα/Nrf2 signaling pathway. Thus, we administered melatonin and isoflurane anesthesia to rat pups at postnatal day 7 (PND 7) and examined whether treatment ameliorated isoflurane-induced oxidative stress and the PKCα/Nrf2 signaling pathway was activated in hippocampus. The results of this study support a novel strategy for treatment of nerve injury induced by isoflurane.

2. Materials and methods

2.1. Animals

The study was approved by the Animal Care and Use of Northeast Agricultural University Committee and was performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals. A total of 16 pregnant female Wistar rats, provided by Harbin Medical University Experimental Animal Center, and were housed in standard polypropylene cages liberally lined with aspen wood shavings. All rats were maintained with 45%–55% humidity in 12 h light/dark cycle and temperature of 22 ± 1°C. The rats were ad libitum accessed to water and food. A total of 72 pups at PND 7 weighing 16–28 g were used for the experiments. Seven-day-old rat pups are at their peak of synaptogenesis (Kentner and Grace, 2017), which is equivalent to the neonatal period in humans (Dobbing and Sands, 1979). Thus, we used 7-day-old pups in our study.

2.2. Exposure to anesthesia

A total of 72 pups (n = 18/group) were randomly assigned into four groups: control (CON) group, rats inhaled 30% O_2 for 4 h; isoflurane (ISO) group, rats were exposed to 1.5% isoflurane plus 30% O_2 for 4 h; isoflurane plus melatonin (I + M) group, melatonin (10 mg/kg) was intraperitoneally administered to pups for 15 min prior to isoflurane inhalation; isoflurane plus solvent (I + S) group, rats were treated with 1% ethanol before isoflurane inhalation. Melatonin (J&K Scientific Ltd., Beijing, China) was dissolved in 1% ethanol solution immediately before administration. The dosage of melatonin was chosen according to a previous study (Mukda et al., 2011). Anesthesia was administered in an anesthesia chamber with 1.5% isoflurane (Yipin Co., Ltd., Hebei, China) mixed with 30% oxygen using an agent-specific vaporizer. Anesthesia was administered using a customized closed-circuit system, and carbon dioxide was eliminated with soda lime.

2.3. Tissue sample preparation

After isoflurane inhalation, three brains per group were immediately removed and fixed in 10% formalin for hematoxylin and eosin (HE), immunohistochemistry (IHC) and immunofluorescence (IF). Three pups from each group were perfused with 0.5% precooled glutaraldehyde, 4% precooled paraformaldehyde and 0.1 M phosphate buffer. Then the hippocampal tissue were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for transmission electron microscopic examination. Six hippocampus per group were dissected out, and stored at -80°C for western. The rest of hippocampus of the pups were used for measurement of ROS, superoxide dismutase (SOD), malonaldehyde (MDA) and glutathione (GSH).

2.4. Measurement of ROS, SOD, MDA and GSH

The levels of ROS and MDA, and the activity of SOD and GSH were measured according to the previous study (Du et al., 2017). Each sample was repeated in triplicate.

2.5. Observation of histopathology

Once the hippocampus was fixed, dehydration of samples was carried out in a graded ethanol series, and then the samples were embedded in paraffin (Yu et al., 2015). Paraffin-embedded hippocampus tissue was cut into 5-μm sections using a microtome before staining with HE (Zhang et al., 2018). After these procedures, section were observed on microscope (Olympus, Tokyo, Japan) and the camera (Canon, Tokyo, Japan) with the software was used for image capturing.

Hippocampi prepared for transmission electron microscopic examination were dehydrated in a graded ethanol series. Ultrathin sections (40- to 60-nm) were mounted on 200-mesh gold gird and doubled stained with uranyl acetate and lead citrate. Sections were observed with an H-7650 electron microscope.

2.6. Western blotting analysis

After centrifuging hippocampi samples at 12,000 × g for 5 min at 4 °C, supernatants were separated into pre-cooled Eppendorf tubes. Protein concentrations were measured using a bicinchoninic acid method (Beyotime Biotechnology, Jiangsu, China). Samples (30 μg of protein) were separated with SDS-PAGE and proteins were blotted onto a PVDF membrane. Membranes were incubated with anti-Nrf2 (WL02135, 1:750, Wanleibio), anti-PKCα (WL02234, 1:1500, Wanleibio), anti－HO-1 (ab68477, 1:25000, abcam), anti-PCNA