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Effects of propofol and isoflurane on excitatory amino acid carrier 1 mRNA and glutathione protein levels in rat hippocampus

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Abstract

Objective: We compared the effects of two anesthetics, isoflurane and propofol, on the nuclear or cytosolic localization of nuclear factor erythroid 2-related factor 2 (Nrf2), mRNA expression levels of excitatory amino acid carrier 1 (EAAC1), and glutathione (GSH) protein levels in the rat hippocampus.

Methods: Fifty-two adult male Sprague–Dawley rats were randomly divided into three groups: a control group, a group that received propofol for 240 minutes (P240), and a group that received isoflurane for 240 minutes (I240). We compared GSH protein and EAAC1 mRNA expression levels in the rat hippocampus and evaluated Nrf2 content in cytosolic and nuclear fractions in the three groups.

Results: GSH protein and EAAC1 mRNA expression levels were significantly higher in the I240 and P240 groups compared with the control group. The I240 and P240 groups showed lower Nrf2 protein levels in the cytosolic fractions, but higher levels in the nuclear fractions compared with the control group.

Conclusion: Treatment with isoflurane or propofol may enhance GSH production by facilitating translocation of Nrf2 into the nucleus and increasing EAAC1mRNA expression in the rat hippocampus. Isoflurane and propofol show similar profiles in EAAC1 expression-associated GSH production.

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Introduction

Intracellular levels of antioxidants can seriously affect the outcomes of brain injury caused by ischemia, trauma, or hemorrhage.[1–3] Many studies have investigated the possible factors that contribute to improving antioxidant status and minimizing oxidative stress after brain damage or surgery.[4–6] Among these various factors, intracellular glutathione (GSH) plays a major role in the defense mechanism against oxidative stress not only by scavenging reactive oxygen species (ROS), but also by repairing oxidative stress induced-damage of cysteine residues in intracellular proteins.[7,8] Depletion of GSH increases brain damage after an ischemic or traumatic injury.[9–11] GSH depletion causes behavioral or cognitive disorders even in the absence of brain injury.[12,13] However, increased intracellular GSH levels due to treatment with a supplement, such as *N*-acetylcysteine, can reduce brain damage.[14,15] Synthesis of intracellular GSH relies on two ATP-dependent enzymes, glutamate cysteine ligase and GSH synthetase, and is formed by catalysis of three substrates, glutamate, cysteine, and glycine.[7] Among these substrates, cysteine is a rate-limiting substrate in intracellular synthesis of GSH because it is maintained at lower levels compared with the two other amino acids.[16] Therefore, uptake of cysteine into neurons, which mainly involves astrocytes, can be an important factor in determining intracellular GSH levels.[7,17] In neurons, excitatory amino acid carrier 1 (EAAC1) is a main carrier of cysteine.[7] There are five classes of Na^+-dependent excitatory amino acid transporters (EAATs) as follows. Glutamate-aspartate transporter (GLAST or EAAT1) and glutamate transporter 1 (GLT-1 or EAAT2) are distributed mainly in astrocytes. EAAC1 (or EAAT3) is widely expressed in neurons.[18] EAAT4 expression is restricted to cerebellar Purkinje cells and EAAT5 is expressed in the retina.[18,19] EAATs mainly transport glutamate. However, EAAC1 has a unique function of cysteine transport in neurons of the mature brain. EAAC1 expression can be affected by nuclear factor erythroid 2-related factor 2 (Nrf2) expression levels in nuclear fractions.[20,21] Therefore, changes in Nrf2 and EAAC1 expression levels in neurons and uptake of cysteine are important factors for determining intracellular GSH levels.[22]

Prevention of neuronal damage from oxidative stress during brain surgery is crucial for better outcomes. The choice of anesthetic agent between an intravenous drug (i.e., propofol) versus an inhalational agent (i.e., isoflurane) for brain surgery has been commonly based on the effects of cerebral metabolic rate, cerebral blood flow, and autoregulation. Propofol and isoflurane affect the expression of EAAC1.[23–25] However, to the best of our knowledge, the effects of these two anesthetic agents in the mechanism underlying EAAC1 expression and GSH production in the brain have not been compared. Determining the roles of these anesthetic drugs in GSH production in the brain might be informative when choosing an anesthetic agent for reducing oxidative stress.

In this study, we compared the effects of propofol and isoflurane on EAAC1 mRNA and GSH protein levels in rat hippocampus after exposure to either drug for 240 minutes. To determine the underlying mechanism, we compared changes in Nrf2 levels in the cytosolic and nuclear fractions.

Materials and methods

We obtained approval for the study from the Institute Animal Care and Committee of Samsung Biomedical Research Institute (Approval number: 20131031002). The animal experiments were performed according to the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Permission to use propofol in this study was granted by the Korea Ministry of Food and Drug Safety (Permission #1115) under the Act on Narcotics in South Korea.

Animal experiments

Fifty-two adult male Sprague–Dawley rats that weighed 300 to 350 g (Orient Bio, Kyunggi-Do, Korea) were included in this experiment. All rats had a quarantine period of at least 7 days in separate cages and were acclimatized in a temperature-controlled atmosphere with artificial light (12-h/12-h day/night cycle) before the experiment. Food pellets and water were provided *ad libitum*.

The rats were randomly allocated into one of three groups as follows: a control group, a group treated with propofol anesthesia for 240 minutes (P240), and a group treated with isoflurane anesthesia for 240 minutes (I240). EAAC1 expression and GSH synthesis in the hippocampus were compared in rats that received an intravenous anesthetic agent (propofol) and a volatile anesthetic agent (isoflurane). Therefore, achieving equivalent anesthetic depths was important. Consequently, we applied equivalent anesthetic doses of isoflurane and propofol for the equivalent anesthetic depths, according to a previous study.[26] To maintain the depth of anesthesia in our study, we used a modified method of stimulating the genioglossus muscle from that of the previous study. The details of this method are as follows. Immediately after intubation, we inserted an insulated nerve stimulator needle (B. Braun, Stimuplex® D; B Braun, Melsungen, Germany) into the genioglossus muscle by using an otoscope. We then stimulated the genioglossus muscle with 5 mA of four continuous electrical stimulations at a frequency of 1 cycle/s every 30 minutes. In responders, we raised isoflurane concentrations from 1.6 to 1.7 vol%, and the dose of propofol dose was increased from 800 to 850 μg/kg/minute. In non-responders, we lowered isoflurane concentrations from 1.7 to 1.6 vol% and the propofol dose was decreased from 850 to 800 μg/kg/minute. General anesthesia with isoflurane (Forane®; Ilsung Pharmaceutical, Seoul, Korea) in the I240 group was maintained at a concentration of 1.6 to 1.7 vol%. Rats that were allocated to the P240 group were briefly anesthetized with isoflurane, and then treated with a continuous infusion of propofol (Fresofol; Fresenius-Kabi, Hessen, Germany), which was initiated immediately after placement of a 24 gauge angiocath in the tail vein. Propofol infusion was maintained at a rate of 800 to 850 μg/kg/minute in the P240 group after three or four bolus injections of propofol (2500 μg). All of the rats in the P240 and I240 groups were mechanically ventilated (Harvard Apparatus, Holliston, MA, USA) at a respiratory rate of 70 to 75 breaths/minute with a fraction of inspired oxygen of 0.3. The end-tidal CO_2 was monitored and maintained within the range of 35 to 40 mm Hg (Ohmeda Excel 210SE anesthetic machine; Datex Instrumentarium Corp., Helsinki, Finland). The rectal temperature of rats in all groups was maintained within

37.0°C ± 0.5°C. The hippocampi in the control group were harvested from rats that had been briefly anesthetized with isoflurane in a sealed chamber. The rats in the I240 and P240 groups were sacrificed, and the hippocampi were harvested at the end of the experiment.

Western blotting

Protein extractions of cytosolic and nuclear fractions were performed using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (NER and CER; ThermoFisher Scientific Inc. Waltham, MA, USA) according to the manufacturer's recommendation. Briefly, rat hippocampi in all groups were homogenized in an appropriate volume of CER I, vortexed vigorously for 15 s, and then combined with ice-cold CER II reagent. After vortexing for 5 s, the extracts were incubated on ice for 1 minute, followed by centrifugation at $16{,}000 \times g$ to obtain the cytoplasmic fraction of proteins. The insoluble pellets were suspended with ice-cold NER, vortexed vigorously for 15 s, and incubated on ice for 40 minutes with vortexing every 10 minutes. The samples were centrifuged at $16{,}000 \times g$ to obtain the nuclear fractions of protein. Equal amounts of cytosolic and nuclear proteins (30 μg) in the three groups were determined by the Lowry method and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 4% to 15% gradient gels (Bio-Rad, Hercules, CA, USA). The gels were then transferred onto polyvinylidene difluoride filter membranes (Millipore, Bedford, MA, USA). After blocking with 5% skim milk in Tris-buffered saline (TBS), the membranes were incubated overnight (14 hours) at 4°C with anti-Nrf2 primary antibody (Cat#: sc-722; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS buffer with 5% skim milk. The membranes were washed three times with TBS with 0.5% Tween 20 and incubated again with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA) at room temperature for 2 hours. The membranes were washed three times with TBS with 0.5% Tween 20 and once with TBS. The blots were treated with ECL solution (Promega, Madison, WI, USA) and exposed to medical X-ray film (Agfa Healthcare, Mortsel, Belgium) for 1 to 10 minutes. Western blot analyses were performed in triplicate to confirm the results.

Determination of glutathione levels in the hippocampus

To analyze hippocampal GSH levels in each group, GSH measurements were performed using the GSH-Glo™ Glutathione Assay (Promega). Briefly, seven rats in each group were perfused intracardially with 300 mL of cold phosphate-buffered saline (PBS) containing 5 U/mL of heparin, and the hippocampus from each rat was harvested as quickly as possible. The hippocampus was homogenized in cold PBS containing 2 mM EDTA at a ratio of 10 mg of hippocampus/2 mL of PBS with 2 mM EDTA. A volume of 50 μL of GSH-Glo™ reagent was then added to 50 μL of tissue supernatant in 96-well plates and incubated at room temperature for 30 minutes. A volume of 100 μL of Luciferin Detection reagent (Promega) was added and incubated with gentle shaking for 15 minutes. Finally, luminescence measurements were performed.

Immunohistochemistry

Immunohistochemical staining was performed as described previously.[27] The rats were deeply anesthetized with 4% isoflurane in 100% oxygen and perfused transcardially with 250 mL of 0.9% normal saline. This was immediately followed by 4% paraformaldehyde in 0.1 M PBS for 5 minutes. The harvested brains were

post-fixed for 12 hours in 4% paraformaldehyde and immersed in 20% sucrose in 0.1 M PBS overnight. The brains were then rapidly frozen in liquid nitrogen and stored at −80°C until use for immunohistochemistry. The brains were sectioned coronally on a cryostat at a thickness of 20 μm. Before immunostaining, the brain sections were rinsed three times with 0.1 M PBS for 5 minutes, and nonspecific protein binding was blocked using blocking buffer (2% horse serum, 0.2% Triton X-100, and 0.1% bovine serum albumin in 0.1 M PBS) at 4°C for 1 hour. The sections were then incubated overnight at 4°C with a primary antibody (EAAC1: Cat# EAAC11-A, Alpha Diagnostic International, San Antonio, TX, USA; Nrf2: Cat# sc-722, Santa Cruz Biotechnology) in 0.1% Triton X-100 in 0.1 M PBS. After washing with 0.1 M PBS three times, the sections were incubated with the corresponding Alexa Fluor-conjugated IgG secondary antibody (Alexa Fluor 488 and 546 ThermoFisher Scientific Inc.) at a dilution of 1:200 for 2 hours at room temperature. After three washes, the sectioned brain tissues were mounted with anti-fade medium containing 4′-6-diamidino-2-phenylindole (DAPI) for nuclei staining (Vectashield; Vector Laboratories, Burlingame, CA, USA). Fluorescence images of the rat hippocampus CA3 area in each group were acquired using a Zeiss fluorescence microscope (Zeiss, Oberkochen, Germany).

Quantitative real-time reverse transcription-polymerase chain reaction

Under deep anesthesia by inhalation, seven hippocampi from each group were rapidly collected and immediately frozen in liquid nitrogen. EAAC1 mRNA was measured by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). The tissues were homogenized and total RNA was extracted using TRI reagent (T9424; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. The primer Taqman probes for rat EAAC1 and Nrf2 (Rn00564705_m1 and Rn00477784_m1, respectively, ThermoFisher Scientific) were used. For qRT-PCR, 2 μg of total RNA was reverse-transcribed by Moloney murine leukaemia virus reverse transcriptase (Promega), and 2 μL of the reverse transcription product was used as the polymerase chain reaction (PCR) template. qRT-PCR was performed using iQ SYBR Green SuperMix and the iCycleriQTM Real-time PCR Detection System (Bio-Rad). The PCR conditions consisted of denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s. A dissociation curve was generated at the end of each cycle to verify amplification of a single product. Levels of mRNA were quantified using the $2^{-\Delta\Delta CT}$ method.[28] Expression of mRNA of the target gene was normalized relative to the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of primer pairs of GAPDH (Gene ID; 24383) were as follows: forward, 5′-GAACATCA TCCCT GCATCCA-3′ and reverse, 5′-CC AGTGAGCTTCCCGTTCA-3′.

Statistical analyses

Statistical analyses were performed using SPSS version 23 software (IBM, Armonk, NY, USA). Data are presented as mean ± standard error. GSH and EAAC1 mRNA expression levels and the intensity ratio of western blotting results were compared using the Kruskal–Wallis test followed by Tukey's test using rank. A P value < 0.05 was considered to indicate statistical significance.

Results

Glutathione levels are significantly increased after 240 minutes of general anesthesia with isoflurane or propofol

We investigated the changes in GSH levels in the rat hippocampus after exposure to isoflurane or propofol for 240 minutes, and analyzed the differences according to the anesthetic agents. GSH levels were significantly higher in the I240 and P240 groups compared with the control group (both $P < 0.05$). However, no significant difference in GSH levels was detected between the I240 and P240 groups (Figure 1).

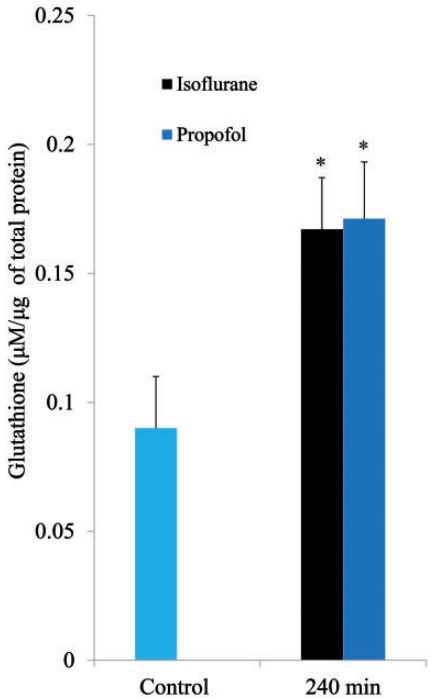

Figure 1. Total glutathione levels in the rat hippocampus. Total glutathione levels were significantly higher after 240 minutes of exposure to isoflurane (I240) or propofol (P240) in the rat hippocampus compared with the control group. However, increased glutathione levels showed no significant difference between the I240 and P240 groups. *$P < 0.05$, compared with the control group.

EAAC1 mRNA expression levels are significantly increased after 240 minutes of general anesthesia with isoflurane or propofol

After confirming that GSH levels were significantly increased in the I240 and P240 groups, we investigated whether EAAC1 and Nrf2 mRNA expression levels were increased in parallel to the increase in GSH levels. Analysis of qRT-PCR showed that EAAC1 mRNA expression levels were significantly higher in the I240 and P240 groups compared with the control group (both $P < 0.05$). However, there was no significant difference in EAAC1 mRNA expression levels between the I240 and P240 groups (Figure 2). Total Nrf2 mRNA expression did not show any significant difference in the I240 and P240 groups compared with the control group.

Nrf2 protein levels are significantly decreased in the cytosolic fractions, but increased in the nuclear fraction after 240 minutes of general anesthesia with isoflurane or propofol

Nrf2 is involved in increasing EAAC1 expression after translocating into the nucleus from the cytosol. Therefore, we performed western blot analyses to evaluate the changes in Nrf2 protein in the cytosolic and nuclear fractions after 240 minutes of general anesthesia with isoflurane or propofol. After 240 minutes of exposure to isoflurane or propofol, Nrf2 protein levels in the cytosolic fractions of rat brains were significantly lower compared with those in controls (both $P < 0.05$) (Figure 3). However, Nrf2 protein levels in the nuclear fraction were significantly higher in the I240 and P240 groups than in the control group (both $P < 0.05$).

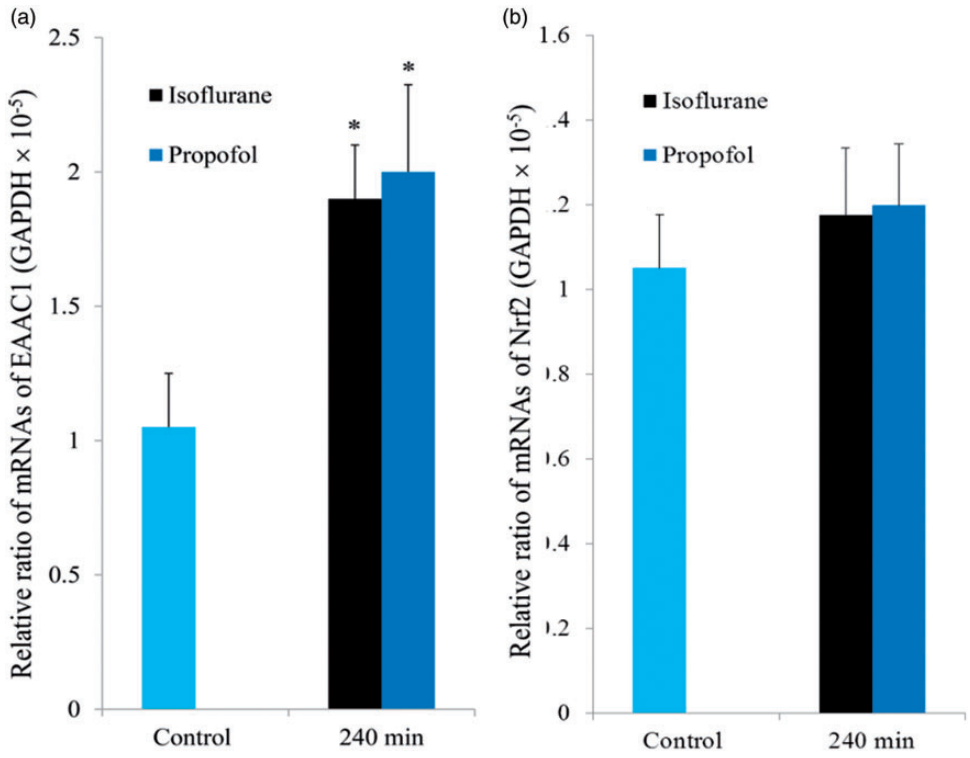

Figure 2. EAAC1 (a) and Nrf2 mRNA expression levels (b). EAAC1 mRNA expression levels were significantly upregulated after 240 minutes of exposure to isoflurane (I240) or propofol (P240) compared with the control group. EAAC1 mRNA expression levels were not significantly different between the I240 and P240 groups. $^{*}P < 0.05$, compared with the control group. However, total Nrf2 mRNA expression levels did not show a significant difference between the I240 and P240 groups. EAAC1: excitatory amino acid carrier 1; Nrf2: nuclear factor erythroid 2-related factor 2.

Immunohistochemical analysis of Nrf2 in rat brains after 240 minutes of exposure to isoflurane or propofol shows nuclear expression in neurons in the CA3 area of rat hippocampus

Immunohistochemical analyses showed that cell surface expression of EAAC1 in neurons in the CA3 area of the rat hippocampus was higher in the I240 and P240 groups compared with the control group, which is consistent with the qRT-PCR results. The control group showed a small amount of Nrf2 in the nuclei. However, after 240 minutes of exposure to isoflurane or propofol, higher levels of Nrf2 were observed in the neuronal nuclei in the area of the rat hippocampus in the I240 and P240 groups than in the control group (Figure 4).

Discussion

During oxidative stress, intracellular GSH content can be an important factor in determining the extent and severity of neuronal damage.[7] We compared the effects of two anesthetic agents on production of intracellular GSH to determine whether one agent provides better intraoperative neuroprotection than the other agent. The present study showed that 240 minutes of exposure to

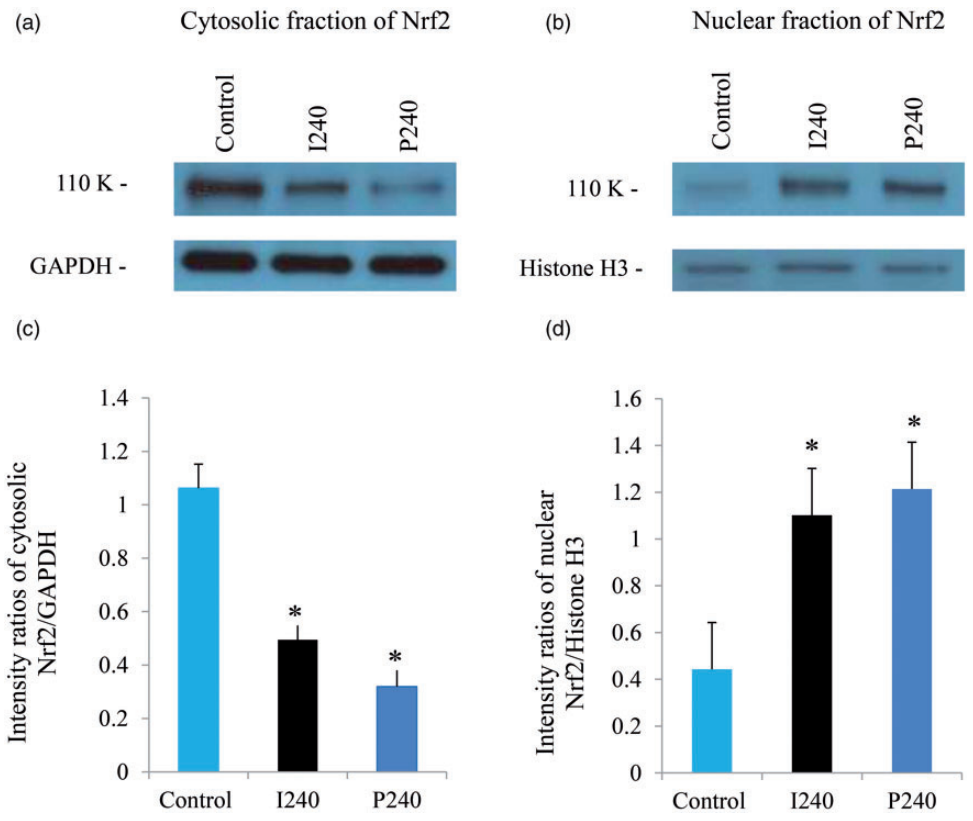

Figure 3. Western blot analysis of cytosolic and nuclear fractions of Nrf2. Nrf2 levels were lower in the cytosolic fraction after 240 minutes of general anesthesia with isoflurane (I240) or propofol (P240) compared with the control group. In contrast, Nrf2 levels were higher in the nuclear fraction in the I240 and P240 groups compared with the control group. Densitometric quantification of western blot results of (a) and (b) are shown in (c) and (d), respectively. $^{*}P < 0.05$, compared with the control group. Nrf2: nuclear factor erythroid 2-related factor 2.

isoflurane or propofol increased Nrf2 translocation into the nucleus and caused a significant increase in EAAC1 mRNA expression and GSH production levels in the rat hippocampus compared with controls. However, EAAC1 mRNA expression and GSH production levels were not significantly different between the I240 and P240 groups.

Propofol is an intravenous anesthetic agent. Propofol shows mitigating effects on oxidative stress by scavenging peroxynitrite, improving the cellular oxidative defense mechanism by upregulating heme oxygenase-1,[29] and reducing ROS production from mitochondria.[30,31] Pre- or post-conditioning by isoflurane has neuroprotective effects by inhibiting ROS release from the mitochondria[32] or by increasing inducible nitric oxide synthase.[33] In addition to these mechanisms, our results indicated that propofol and isoflurane showed similar effects in facilitating translocation of Nrf2 into the nucleus from the cytosol. This in turn increased EAAC1 mRNA expression and GSH production levels. Under normal conditions, the transcription factor Nrf2 is rapidly degraded by

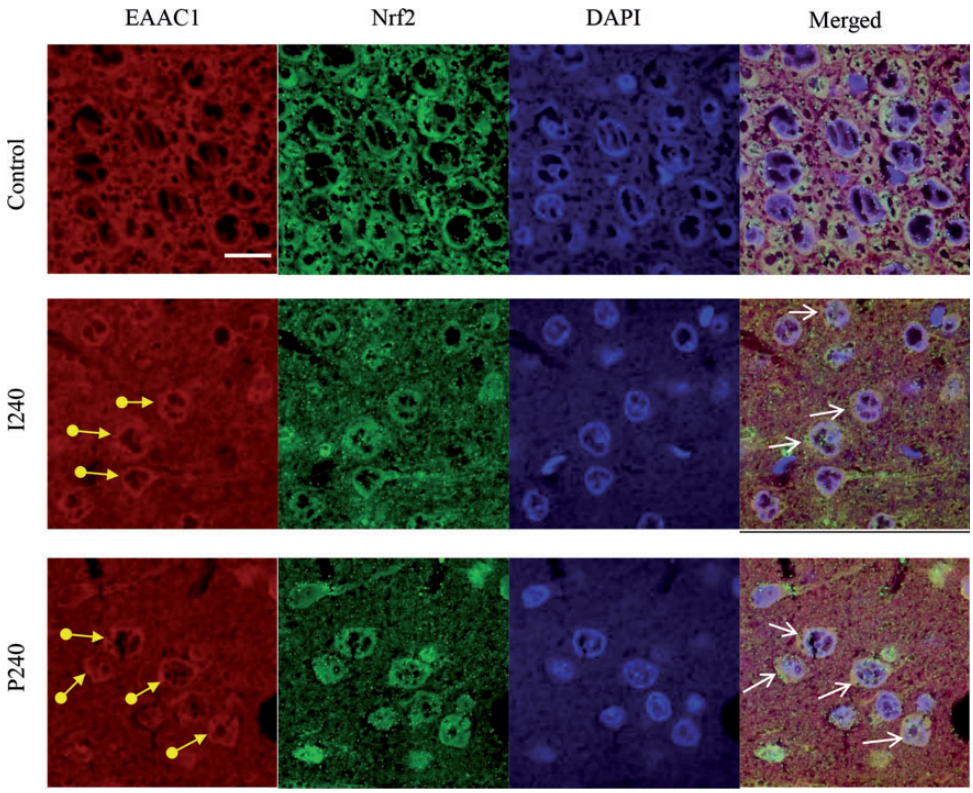

Figure 4. Immunohistochemical analyses of EAAC1 and Nrf2. Exposure for 240 minutes to isoflurane (I240) or propofol (P240) increased the cell surface expression of EAAC1 (yellow arrows). Only a small amount of Nrf2 was detected in the nucleus in the control group. However, Nrf2 was increased in the neuronal nucleus after 240 minutes of exposure to isoflurane or propofol (white arrows). Scale bar: 50 μm. EAAC1: excitatory amino acid carrier 1; Nrf2: nuclear factor erythroid 2-related factor 2.

Kelch-like ECH-associated protein-mediated ubiquitylation in the cytosol. However, in the presence of oxidative stress or chemical stimulation, proteosomal degradation of Nrf2 in the cytosol subsides, and Nrf2 translocates from the cytosol into the nucleus,[34] where it activates Nrf2 antioxidant response elements. Escartin et al.[21] reported that chemical stimulation by *tert*-butylhydroquinone increases expression of Nrf2, which results in enhanced Nrf2 antioxidant response element pathway activation in the nucleus and increased neuronal GSH synthesis by upregulated EAAC1 expression. In our study, total Nrf2mRNA expression did not show significant changes in the I240 and P240 groups compared with the control group. However, western blotting and immunohistochemical analyses in our study showed that Nrf2 protein levels were decreased in the cytosolic fractions, but increased in the nuclear fractions in the I240 and P240 groups. Additionally, EAAC1 mRNA expression was significantly upregulated in the I240 and P240 groups compared with the control group. Our results suggested that isoflurane and propofol did not cause increased expression of Nrf2, but these anesthetic agents might facilitate translocation of Nrf2 from the neuronal cytosol into the nucleus after 240 minutes

of exposure. The increased translocation of Nrf2 in the neuronal nucleus in the rat hippocampus likely caused increased EAAC1 mRNA expression in our study. In mature neurons, EAAC1 replaces the role of the cystine/glutamate antiporter system xc^-, which is expressed in immature neurons, and assumes the role as the main route for uptake of cysteine.[7,21] This finding is supported by several studies in which EAAC1 knockout mice showed reduced neuronal GSH content, increased vulnerability to oxidative stress, and increased neuronal death.[35,36] Therefore, EAAC1 expression levels can be an important factor in determining the intraneuronal GSH content level in mature neurons. In our study, we detected increased intraneuronal GSH protein and EAAC1 mRNA expression levels in the rat hippocampus after 240 minutes of exposure to isoflurane or propofol compared with controls. However, no significant differences in GSH content and EAAC1 mRNA expression levels were detected between the I240 and P240 groups. This finding suggests that use of propofol or isoflurane leads to similar intraoperative neuroprotection effects because they show similar EAAC1 mRNA and GSH protein levels after exposure to anesthesia for 240 minutes. From these results, we speculate that exposure to isoflurane or propofol for 240 minutes may facilitate translocation of Nrf2 from the cytosol into the nucleus. Furthermore, isoflurane and propofol may have a role in increasing EAAC1 expression, which causes increased intraneuronal GSH content in the rat hippocampus. These effects of isoflurane and propofol might contribute to the defense mechanism mitigating oxidative stress during the intraoperative period.

This study has several limitations. Therefore, our results should be interpreted with caution. First, the control group breathed only room air. However, the P240- and I240-treated groups were ventilated with a fraction of inspired oxygen of 0.3, which is a higher content than that in room air. Second, although we maintained equivalent anesthetic depths based on the methods of a previous study that investigated genioglossus muscle activity[26], we cannot exclude the possibility that the anesthetic depths differed between the P240 and I240 groups during the experimental period. Third, the 240-minute period of exposure to anesthetic agents in rats was relatively long and the anesthetic depth using either isoflurane or propofol was relatively deep. Therefore, the effect of isoflurane or propofol on Nrf2 translocation and EAAC1 mRNA and GSH protein levels might not be as marked with shorter time exposures and a light anesthetic depth.

In conclusion, exposure to isoflurane and propofol for 240 minutes leads to enhanced GSH production by facilitating the translocation of Nrf2 into the nucleus and increasing expression of EAAC1 in the rat hippocampus. These effects likely act as a defense mechanism against oxidative stress during the intraoperative period. However, there are no significant differences in EAAC1 mRNA expression and GSH protein levels in rat hippocampal neurons between exposure to isoflurane and propofol for 240 minutes. Therefore, isoflurane and propofol appear to have equivalent effects in GSH production-associated protection against oxidative stress.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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