



Neuropharmacology and analgesia

Midazolam inhibits the hypoxia-induced up-regulation of erythropoietin in the central nervous system

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ABSTRACT

Erythropoietin (EPO), a regulator of red blood cell production, is endogenously expressed in the central nervous system. It is mainly produced by astrocytes under hypoxic conditions and has proven to have neuroprotective and neurotrophic effects. In the present study, we investigated the effect of midazolam on EPO expression in primary cultured astrocytes and the mouse brain. Midazolam was administered to 6-week-old BALB/c male mice under hypoxic conditions and pregnant C57BL/6N mice under normoxic conditions. Primary cultured astrocytes were also treated with midazolam under hypoxic conditions. The expression of EPO mRNA in mice brains and cultured astrocytes was studied. In addition, the expression of hypoxia-inducible factor (HIF), known as the main regulator of EPO, was evaluated. Midazolam significantly reduced the hypoxia-induced up-regulation of EPO in BALB/c mice brains and primary cultured astrocytes and suppressed EPO expression in the fetal brain. Midazolam did not affect the total amount of HIF proteins but significantly inhibited the nuclear expression of HIF-1 α and HIF-2 α proteins. These results demonstrated the suppressive effects of midazolam on the hypoxia-induced up-regulation of EPO both *in vivo* and *in vitro*.

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1. Introduction

Erythropoietin (EPO) is a widely recognized hematopoietic growth factor primarily produced in the kidneys and fetal liver (Fisher, 2003). EPO expression has also been found in several other tissues, including the central nervous system (CNS) (Marti et al., 1996; Takahashi et al., 1994). Although neurons and endothelial cells are possible sources of EPO expression, astrocytes are considered to be the main source of EPO in CNS (Masuda et al., 1994; Ruscher et al., 2002; Weidemann et al., 2009). CNS-derived EPO is induced in an oxygen tension-dependent manner (Noguchi et al., 2007) and is thought to ameliorate hypoxia-induced brain damage by inhibiting neuronal apoptosis (Siren et al., 2001), suppressing proinflammatory cytokines (Yamasaki et al., 2005), and reducing glutamate release (Chen et al., 2007). In addition, EPO and its receptor are expressed in CNS during mammalian fetal development (Yu et al., 2002) and remain markedly high compared with those in adults under physiological conditions (Liu et al., 1997). In fact,

various studies of EPO- and EPO receptor-deficient mice have shown EPO to be essential for neuronal development (Lin et al., 1996; Wu et al., 1995).

Recently, we found that the volatile anesthetic isoflurane suppressed the induction of EPO expression in the mouse brain and cultured astrocytes in a concentration- and time-dependent manner (Tanaka et al., 2011). Although anesthetics are a common component of surgical procedures, they are also used for sedation in the intensive care unit (ICU). In addition, drug exposure tends to be longer in ICU sedations than in surgical procedures. Furthermore, global or regional brain hypoxia could occur in the critically ill patients in ICU (Malagon et al., 1996). Benzodiazepines, particularly midazolam, are widely used for sedation, particularly in children (Rappaport et al., 2011). Therefore, the effect of midazolam on EPO expression in CNS may warrant a modification of clinical management. To date, the effect of midazolam on CNS-resident EPO remains poorly studied. The aim of the present study was to clarify whether midazolam affects the hypoxia-induced up-regulation of EPO in the fetal and mature mouse brain and in the primary culture of astrocytes.

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2. Materials and methods

2.1. Animals

All experimental procedures were conducted in accordance with guidelines from the Institutional and National Institutes of Health and performed with the authorization of the Animal Research Committee, Graduate School of Medicine, Kyoto University (Permit Number: 14511). C57BL/6N pregnant mice or 6-week-old male BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed in sawdust-lined cages with *ad libitum* feeding under 12-h light–dark cycles and constant temperature (24 °C) conditions. Every effort was made to lessen the number of animals used and to alleviate any pain and suffering experienced by the animals.

2.2. Chemicals and drugs

Midazolam (PubChem CID: 4192), flumazenil (PubChem CID: 3373), and colchicine (PubChem CID: 6167) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Clonazepam (PubChem CID: 2802) and cytochalasin (PubChem CID: 5311281) was obtained from Wako (Osaka, Japan), and ciliobrevin (PubChem CID: 6883982) was purchased from Merck (Whitehouse Station, NJ, USA). PK11195 (PubChem CID: 1345) was obtained from Adipogen (San Diego, CA, USA). Clonazepam was dissolved in ethanol, and all other drugs were dissolved in dimethyl sulfoxide. Oxygen (O₂) and nitrogen (N₂) gas were acquired from Taiyo Nippon Sanso (Tokyo, Japan).

2.3. Cell culture

The primary culture of astrocytes from 1-day-old C57BL/6N-CrSlc mice was isolated according to a previously described method (Tanaka et al., 2011). Cerebral cortices were dissociated by filtering with a 320 µm sieve. The cortices were treated with trypsin and DNase (20 min), centrifuged at 1200 rpm (5 min), and filtered with a 100 µm sieve. Following this, the cortices were placed in a cell-cultivation flask with 50 ml of a prepared medium, which consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.1 mg ml⁻¹ streptomycin, 100 U ml⁻¹ penicillin, and 10% fetal bovine serum. Cultures were maintained in a temperature-controlled incubator. The culture media were changed twice a week, and each time the medium was changed, a shaking procedure removed the oligodendrocytes and their precursors from the media.

2.4. Hypoxic cell treatment

The cell cultures were placed into a dedicated CO₂ multi-gas incubator (APM-30DR; Astec, Fukuoka, Japan) to continuously expose the cells to 1% O₂ + 94% N₂ + 5% CO₂ for indicated durations.

2.5. Hypoxic or hyperoxic treatment of mice

Mice were subjected to a previously described hypoxic treatment (Tanaka et al., 2011). Mice were placed in plastic chambers. Following this, an anesthetic delivery system (Custom50; Aika, Tokyo, Japan) released a mixed gas that contained N₂ and O₂ into the chamber. A gas monitoring system (Capnomac Ultima Anesthesia Monitor; Ohmeda, Helsinki, Finland) continuously measured the O₂ and CO₂ concentrations. The sedative dose of midazolam in rodents for intraperitoneal administration was reported in the range of 1 and 10 mg kg⁻¹ (Kissin et al., 1990); however, in another report (Inada et al., 2004), 25 mg kg⁻¹ was used for light sedation and 50 mg kg⁻¹ was used for deep sedation. For deciding

the appropriate dose of midazolam under hypoxic conditions, the mice were intraperitoneally injected with a light sedative dose of midazolam (20 mg kg⁻¹) and exposed to 10% O₂ for 3 h. The degree of sedation was determined according to the rating scale in the previous study (Boast et al., 1988). Righting reflexes under the hypoxic condition with midazolam were almost score +1 (wobbling gait during locomotion, but the mouse rights itself within 2 s) until 3 h in every 30 min; therefore, we decided the dose of midazolam to be 20 mg kg⁻¹. The mice were given 1 h to adapt to the hypoxic conditions. Immediately after the 1 h adaptation period, intraperitoneal injection of midazolam (20 mg kg⁻¹) was given. Flumazenil (20 mg kg⁻¹) was intraperitoneally administered immediately after the injection of midazolam. Following the injections, the mice were exposed to 10% O₂ for 3 h. Pregnant C57BL/6Ncr mice were subjected to hyperoxic treatment, which was similar to the hypoxic treatment method. The mice were given 1 h to adapt to the hyperoxic conditions. Following the injections, the mice were exposed to 100% O₂ for 3 h. After the mice were exposed to 10% or 100% O₂, euthanasia by cervical dislocation was performed. The brains of the mice that were used in the hypoxic experiments were rapidly removed and stored at –80 °C. The pregnant mice had their pups delivered by cesarean sections. The fetal brains were quickly removed and stored at –80 °C.

2.6. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

NucleoSpin[®] RNA kits (Macherey-Nagel, Düren, Germany) were used to isolate RNA from fetal and mature mice brains and from the primary culture of postnatal mouse astrocytes. First-strand cDNA synthesis was performed using the One Step SYBR[™] PrimeScript[™] RT-PCR Kit I (Takara Bio, Shiga, Japan) according to the manufacturer's protocols. qRT-PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems, CA, USA). The 18S rRNA internal standard was used to calculate the relative fold changes for each target expression of mRNA. Primers for 18S, glucose transporter 1 (GLUT1), and vascular endothelial growth factor (VEGF) were obtained from Qiagen (catalog number QT01036875, QT01044953, and QT00160769, respectively; Valencia, CA, USA). The forward and reverse EPO primers (Life Technologies, Tokyo, Japan) were 5'-CTGGGAGCTCA-GAAGGAATTGATG-3' and 5'-CCGGAGGAAGTTGGCGTAGAC-3', respectively, whereas the forward and reverse lactate dehydrogenase A (LDHA) primers (Takara Bio) were 5'-GGATGAGCTTGCCCTTGTGA-3' and 5'-GACCAGCTTGGAGTTCGCAGTTA-3', respectively.

2.7. EPO ELISA

The supernatants of the astrocyte cell cultures were assayed using an ELISA kit (R&D Systems Europe, Abingdon, UK), according to the manufacturer's protocols to quantitatively evaluate the concentration of EPO.

2.8. Immunoblot assay

A previously described method was used to isolate whole-cell lysates of primary cultured astrocytes (Tanaka et al., 2011). Cells were harvested and washed with phosphate-buffered saline and then centrifuged at 1500 rpm for 5 min. Cell pellets were resuspended in an ice-cold lysis buffer composed of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 2 mM DTT, 5 mM EDTA, 1 mM sodium orthovanadate, and complete protease inhibitors[™] (Roche Diagnostics, Tokyo, Japan). The lysed cells were centrifuged at 15,000 rpm for 10 min at 4 °C, following which the supernatant that contained the

whole-cell lysates was collected. The Nuclear Extraction Kit (Active Motif, Carlsbad, CA, USA) was used to isolate nuclear extracts from the primary cultured astrocytes. Proteins (100 μ g each) from these samples were separated with a 7.5% SDS-polyacrylamide gel and immunoblotted using a previously described method (Tanaka et al., 2011). Immunoblots were incubated in a 1:1000 dilution of the following primary antibodies: rabbit polyclonal anti-HIF-1 α (10006421, Cayman Chemical, Ann Arbor, MI, USA), rabbit polyclonal anti-HIF-2 α (NB100-122, Novus Biologicals, Inc., Littleton, CO, USA), mouse monoclonal anti-HIF-1 β (#611078, BD Biosciences, San Jose, CA, USA), rabbit polyclonal anti-lamin A/C (#2032; Cell Signaling, Stockholm, Sweden), and mouse monoclonal anti- β -actin (A5316, Sigma-Aldrich).

2.9. Dynein ATPase activity assay

Cytoplasmic dynein purified from the porcine brain was purchased from Cytoskeleton, Inc. (CS-DN01, Denver, CO, USA). ATPase activity was measured using the ELIPA Biochem Kit (BK054, Cytoskeleton). Each reaction contained 0.8 μ g of dynein, 2.0 μ M taxol-stabilized microtubules (T240, Cytoskeleton), 11 μ M paclitaxel (Txd01, Cytoskeleton), 12 mM PIPES (pH 7.0), and 5 mM MgCl₂, which was prepared in half-area 96-well plates. Reactions were initiated by the addition of ATP (A3377, Sigma-Aldrich) to yield a final concentration of 0.19 mM. The absorbance of each reaction mixture was measured at 650 nm, the background subtraction (reaction buffer without microtubules or dynein) was attained, and the absorbance with corrected background values was plotted. Data represent the average of three independent reactions.

2.10. Statistical analysis

All the data for the continuous variables are presented as mean $\pm$ standard deviation (SD). The differences between each group were tested by one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test. *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Midazolam inhibits the expression of EPO mRNA in the fetal and mature mouse brain

To examine the *in vivo* effect of midazolam on EPO expression in the brain, we exposed 6-week-old BALB/c mice to hypoxic conditions (10% O₂) for 3 h with and without midazolam. Hypoxic exposure significantly increased EPO mRNA expression, while midazolam (20 mg kg⁻¹) suppressed hypoxia-induced EPO mRNA expression in the mouse brain (Fig. 1A). A simultaneous injection of flumazenil did not prevent the suppression of EPO expression (Fig. 1A). Because CNS-derived EPO is essential for neurogenic

development, the effect of midazolam on EPO expression in the fetal mouse brain was evaluated. A fetal brain is physiologically hypoxic because the blood in the umbilical vein going to the fetus

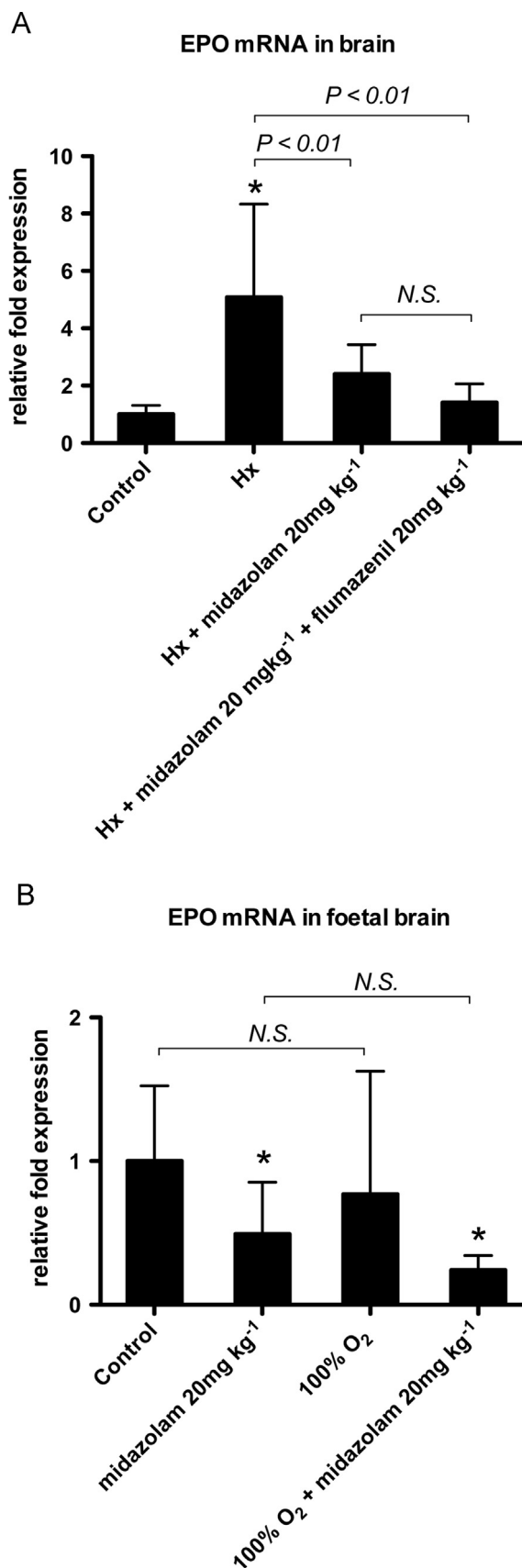


Fig. 1. Effect of midazolam on erythropoietin (EPO) expression in the mouse brain. (A) Six-week-old BALB/c mice were exposed to 3 h of 10% O₂ (hypoxia: Hx) with and without 20 mg kg⁻¹ midazolam and 20 mg kg⁻¹ flumazenil. Control mice were exposed to 3 h of air without anesthetics. EPO mRNA was obtained from the brain and was assayed using real-time quantitative polymerase chain reaction (qRT-PCR). There were six mice for each treatment condition. (B) C57BL/6N CrSlc mice that were pregnant for 17 days were exposed to 3 h of air or hyperoxic conditions (100% O₂) with and without midazolam. Control mice were exposed to 3 h of air without anesthetics. EPO mRNA that was obtained from the fetal brain was assayed using real-time qRT-PCR. There were six fetuses for each treatment condition. Data are shown as mean $\pm$ SD. The expression levels of 18S mRNA were used as an internal standard to normalize the expression levels of EPO. The relative quantifications to the mean of the control mice are shown. **P* < 0.05 vs. control; N. S., not significant.

is saturated with low PaO_2 . Thus, pregnant C57BL/6N CrSlc mice were exposed to 3 h of air or hyperoxic conditions (100% O_2) with and without midazolam (20 mg kg^{-1}). Midazolam significantly

suppressed EPO mRNA in the fetal brain (Fig. 1B). The suppressive effect of hyperoxia was not detected (Fig. 1B).

3.2. Midazolam inhibits the expression of EPO mRNA and protein in primary cultured astrocytes under hypoxic conditions

An *in vitro* investigation was conducted to exclude the possibility that midazolam-induced hypercapnia or hypotension affected EPO expression. Primary astrocyte cell cultures were exposed to hypoxic conditions (1% O_2) for 4 h with or without midazolam. Hypoxic treatment significantly up-regulated EPO expression, whereas midazolam inhibited the up-regulation in a concentration-dependent manner (Fig. 2A). To verify the effects of midazolam on the EPO protein, the concentration of the EPO protein was determined in the supernatants from the astrocyte culture. Fig. 2B shows that hypoxic exposure for 24 h significantly increased the concentration of the EPO protein, whereas midazolam markedly suppressed the same.

3.3. Effect of drugs stimulating GABA_A receptor or [Translocator Protein (18 kDa): TSPO] on the up-regulation of EPO in the primary astrocyte cell culture of postnatal mice

Midazolam is considered to exert its clinical effect mainly through the GABA_A receptor (Oikkola and Ahonen, 2008); however, flumazenil, a GABA_A receptor antagonist, could not prevent the suppression of EPO by midazolam (Fig. 3A). Midazolam is also known to bind mitochondrial receptors (TSPO) (Papadopoulos et al., 2006), but PK11195, which binds selectively TSPO, did not suppress hypoxia-induced EPO up-regulation (Fig. 3B) or inhibit the effect of midazolam on EPO induction (Fig. 3C). On the other hand, clonazepam, which is a GABA_A receptor-specific ligand with no affinity for TSPO (Sakai et al., 2010), suppressed EPO induction in a concentration-dependent manner (Fig. 3D).

3.4. Effect of midazolam on the hypoxia-inducible factor (HIF)

HIFs are heterodimeric transcription factors that primarily regulate EPO expression in hypoxic conditions (Stockmann and Fandrey, 2006). HIFs are composed of α and β subunits, and the activation of these factors depends on the expression of the α subunit (Semenza et al., 1991). In a normal oxygen environment, the ubiquitin-proteasome pathway rapidly degrades HIF- α ; however, in a low oxygen environment, the α subunit is stabilized and translocated into the nucleus. In the nucleus, the α and β subunits dimerize to form the transcriptionally active HIF heterodimer (Hirota and Semenza, 2005; Wang et al., 1995). The HIF- α family is composed of HIF-1 α , HIF-2 α , and HIF-3 α (Hirota and Semenza, 2005). HIF-1 α and HIF-2 α regulate EPO expression in CNS (Stockmann and Fandrey, 2006). Thus, the effects of midazolam on HIF-1 α and HIF-2 α protein levels were examined by immunoblotting. The primary astrocyte cell cultures were exposed to 4 h of 1% O_2 with and without midazolam and harvested. Hypoxic conditions significantly elevated whole-cell and nuclear HIF-1 α and HIF-2 α proteins levels (Fig. 4). Midazolam did not reduce the

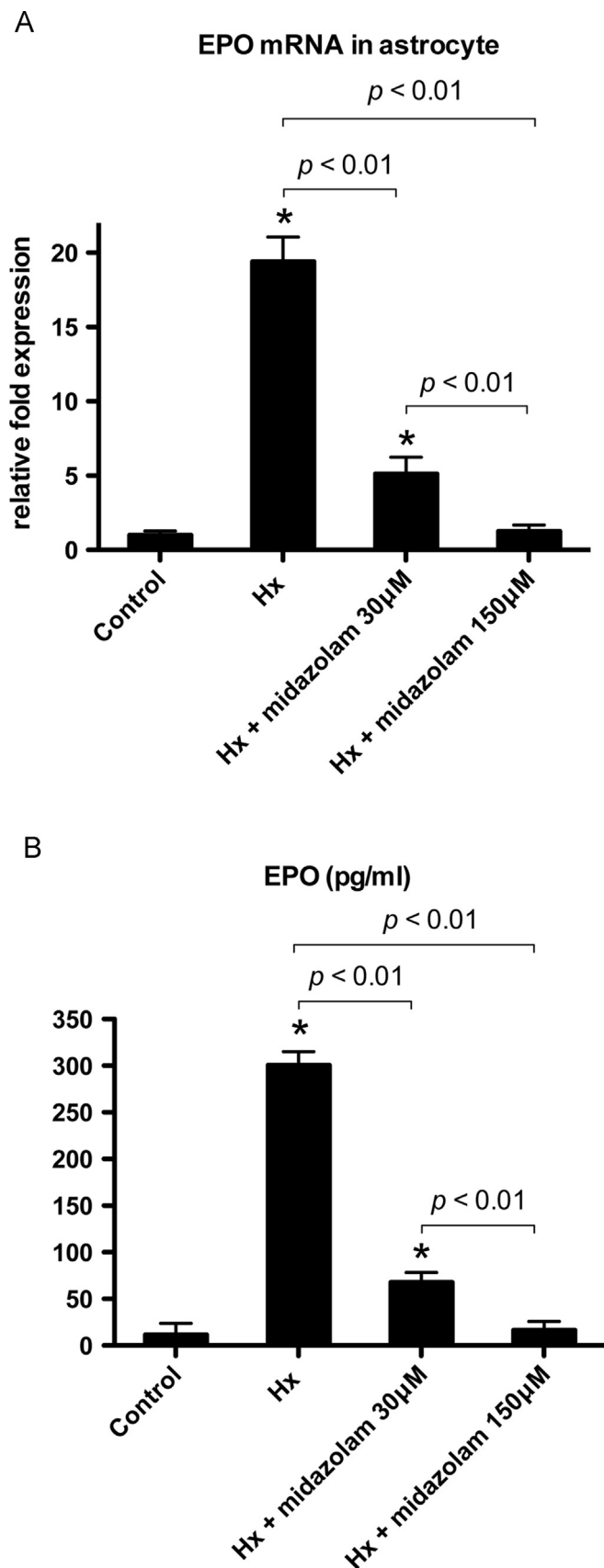


Fig. 2. Effect of midazolam on the up-regulation of erythropoietin (EPO) in the primary astrocyte cell culture of postnatal mice. (A) Primary astrocyte cell cultures were exposed to 4 h of 1% O_2 (hypoxia: Hx) with indicated concentrations of midazolam. Controls were exposed to 4 h of 20% O_2 . EPO mRNA was analysed with real-time qRT-PCR. The expression levels of 18S mRNA were used as an internal standard to normalize the expression levels of EPO. (B) The concentration (pg ml^{-1}) of EPO proteins that were obtained from the primary astrocyte cell culture media was measured using enzyme-linked immunosorbent assay (ELISA). Primary astrocyte cell cultures were exposed to 24 h of 1% O_2 (hypoxia: Hx) with or without midazolam with the indicated concentrations. Controls were exposed to 24 h of 20% O_2 . Data are shown as mean $\pm$ SD ($n=3$); # $P < 0.01$ vs. Hx; * $P < 0.01$ vs. control; N.S., not significant.

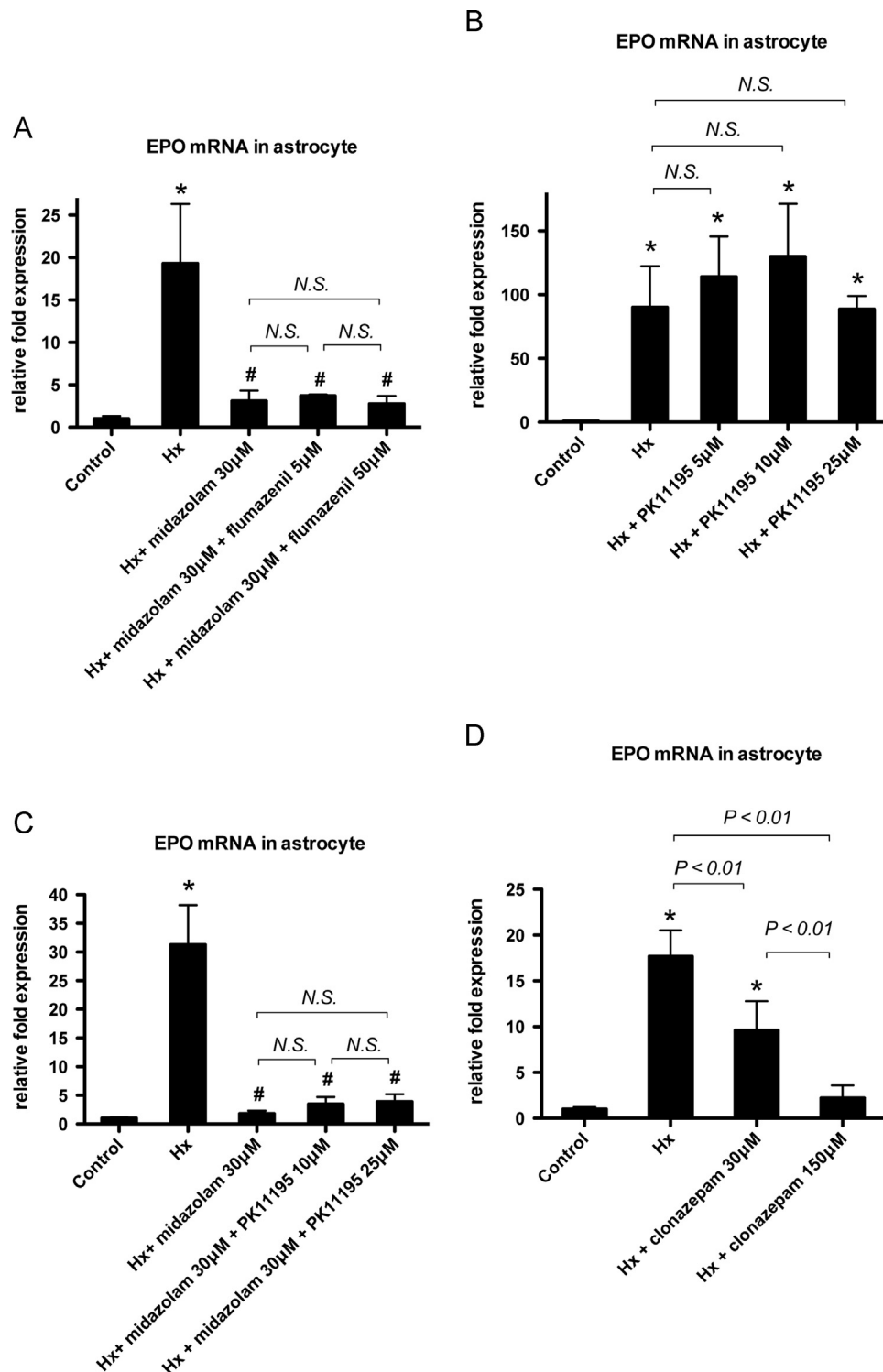


Fig. 3. Effect of drugs stimulating GABA_A receptor or Translocator Protein (18 kDa) on the up-regulation of erythropoietin (EPO) in the primary astrocyte cell culture of postnatal mice. Primary astrocyte cell cultures were exposed to 4 h of 1% O₂ (hypoxia: Hx) with indicated concentrations of (A, C) midazolam, (B) flumazenil, (B, C) PK11195 and (D) clonazepam. Controls were exposed to 4 h of 20% O₂. EPO mRNA was analysed with real-time qRT-PCR. The expression levels of 18S mRNA were used as an internal standard to normalize the expression levels of EPO. Data are shown as mean ± SD (*n* = 3); #*P* < 0.01 vs. Hx; **P* < 0.01 vs. control; N.S., not significant.

HIF- α proteins in whole-cell extracts (Fig. 4A) but suppressed the nuclear levels of HIF-1 α and HIF-2 α proteins (Fig. 4B).

3.5. Effect of midazolam on the expression of the genes regulated by HIF

To confirm the inhibitory effect of midazolam on HIF

transcriptional activity, we investigated their effect on the expression of other HIF target genes: GLUT1, LDHA, and VEGF (Hu et al., 2007). We examined the effect of midazolam on the induction of these genes under hypoxic conditions in primary cultured astrocytes. Hypoxic exposure (1% O₂ for 4 h) induced the expression of GLUT1, LDHA, and VEGF but was significantly suppressed by the administration of midazolam (Fig. 5).

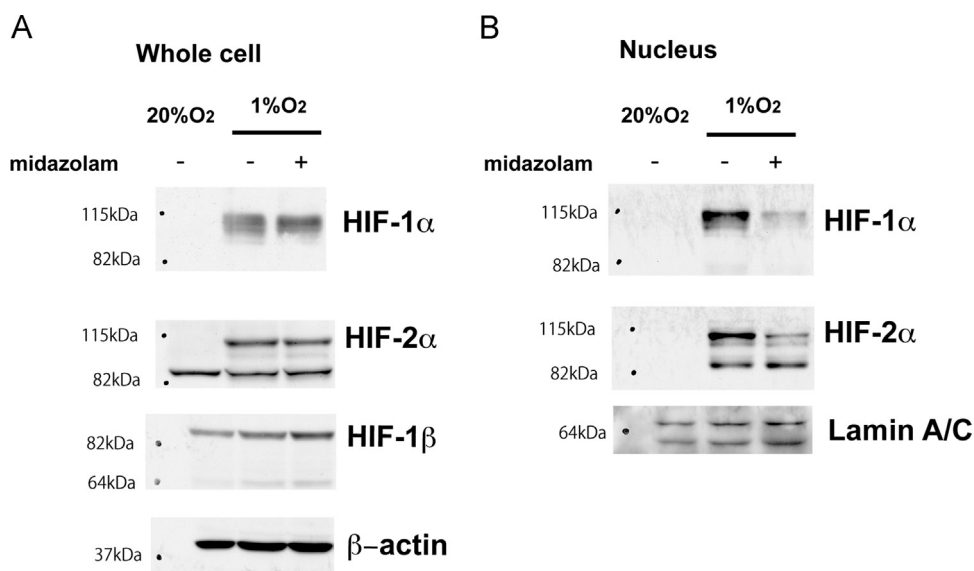


Fig. 4. Immunoblotting measured the expression of the hypoxia-inducible factor (HIF)-1 α and HIF-2 α in whole-cell and nuclear extracts. Immunoblotting measures the expression levels of HIF-1 α and HIF-2 α in (A) the whole-cell and in (B) the nuclear extracts. Primary astrocyte cell cultures were exposed to 4 h of 20% or 1% O₂ with 150 μ M midazolam. These experiments were replicated at least three times and the images in (A) and (B) signify the whole cell and nuclear extract images, respectively.

3.6. Mechanism of midazolam-induced suppression against nuclear accumulation of HIF- α proteins in astrocytes

Fig. 4 shows that midazolam suppressed the accumulation of nuclear HIF-1 α and HIF-2 α proteins; however, the total HIF-1 α and HIF-2 α proteins obtained from the whole-cell extracts remained unchanged. These results suggest that midazolam affects the nuclear translocation of HIF- α proteins. Recent studies have reported that the nuclear translocation of HIF- α proteins depends on the microtubule-based transport system (Carbonaro et al., 2012, 2011). Several cellular cargos are transported by cytoplasmic dynein along the cytoskeletal microtubules toward the cell center (i.e., retrograde transport) (Schiavo et al., 2013; Vallee et al., 2012). Thus, the effects of colchicine, a microtubule polymerization inhibitor; ciliobrevin (Firestone et al., 2012), an ATPase cytoplasmic dynein inhibitor; and cytochalasin, an actin polymerization inhibitor, on EPO expression in astrocytes were investigated. Colchicine (Fig. 6A) and ciliobrevin (Fig. 6B) significantly suppressed the hypoxia-induced expression of EPO, whereas cytochalasin did not (Fig. 6C). Dynein is one of the motor proteins that uses the chemical energy from ATP hydrolysis for mechanical movement (Ori-McKenney et al., 2010). Thus, the effect of midazolam on cytoplasmic dynein activity was evaluated by performing an *in vitro* dynein ATPase activity assay. As shown in Fig. 6D, the relative ATPase activity of cytoplasmic dynein in the presence of microtubules was elevated compared with that of the control reactions of independent microtubules and dynein. Midazolam significantly suppressed dynein ATPase activity (Fig. 6D).

4. Discussion

EPO is the main regulator of haematopoiesis in mammals (Masuda et al., 1994; Ruscher et al., 2002; Weidemann et al., 2009). Although the majority of EPO is peripherally produced, CNS can also produce EPO. Astrocytes followed by neurons and endothelial cells are the main sources of this CNS-derived EPO (Masuda et al., 1994; Ruscher et al., 2002). The EPO receptor is mainly expressed by neurons (Juul et al., 1998). The endogenous secretion of EPO in CNS is oxygen dependent (Noguchi et al., 2007) and has protective effects against hypoxic neuronal damages

(Brines and Cerami, 2005).

In the present study, midazolam suppressed the hypoxia-induced CNS-derived EPO *in vivo* and *in vitro*. Previously, various anesthetics, including isoflurane and propofol, showed similar suppressive effects on the astrocytic expression of EPO during hypoxia (Tanaka et al., 2011). These findings and the results of the present study demonstrate that under hypoxic conditions, clinically used anesthetics suppress EPO expression in CNS. Unfortunately, the effects of anesthetics on astrocytes are not well understood, although previous studies have reported that volatile anesthetics inhibit glutamate uptake (Miyazaki et al., 1997), midazolam stimulates steroidogenesis (Guo et al., 2013), and propofol modulates the expression of aquaporin-4 (Zheng et al., 2008) in astrocytes. However, the results of the present study suggest that various anesthetics have a common biological effect on astrocytes.

Several reports have determined the purpose of EPO in CNS. In a mouse brain stroke model, reduced neurogenesis or impaired neuronal migration was found to occur in mice that lacked the brain-derived EPO receptor (Tsai et al., 2006). A previous study showed that the supplementation of EPO counteracts the hypoxia-induced neuronal death *in vitro* (Liu et al., 2006), whereas another report revealed that an intracerebral injection of EPO reduces the infarction volume and saves the cognitive function of rodent models of ischemia (Siren et al., 2001). Thus, EPO is a neuroprotective molecule that protects the brain against hypoxia and ischemia (Brines and Cerami, 2005; Noguchi et al., 2007). In the present study, we demonstrated that midazolam inhibits EPO mRNA production in primary cultured astrocytes and the mouse brain under hypoxic conditions. Midazolam is commonly administered to critically ill ICU patients, some of whom suffer from systemic or regional hypoxia (Malagon et al., 1996). For example, children with congenital heart disease could have systemic hypoxia, while patients with intracranial diseases may have regional cerebral hypoxia. Considering the pivotal role of EPO in neuroprotection (Milano and Collomp, 2005), midazolam may affect the clinical course of such patients by suppressing EPO production in the brain. However, the present study only investigated brain-derived EPO in a systemic hypoxia model. Therefore, further studies using additional hypoxia models, including ischemia and trauma, are required.

CNS-derived EPO is vital for the development of neuronal

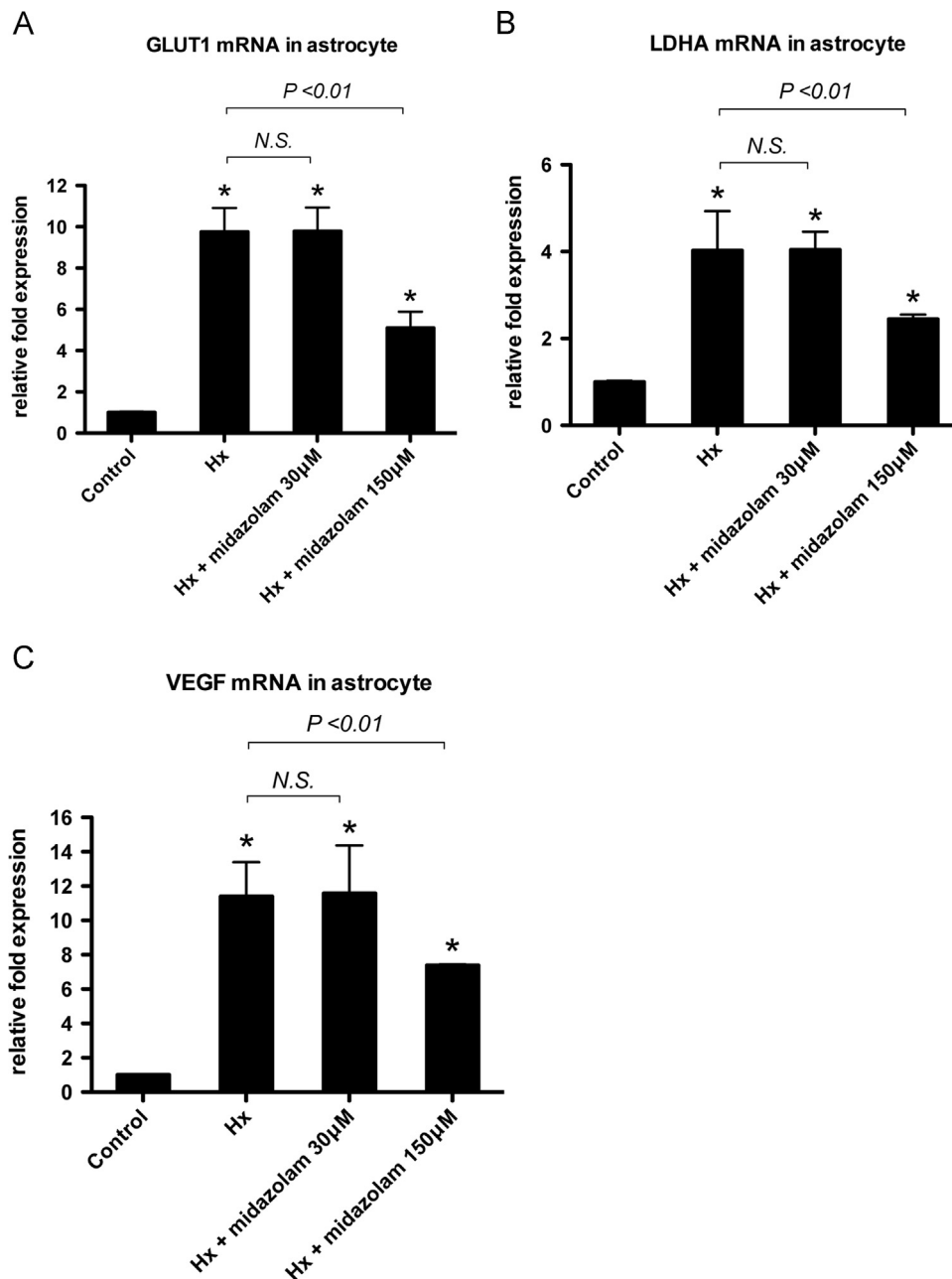


Fig. 5. Effect of midazolam on the expression of the target genes of the HIFs. Primary astrocyte cell cultures were exposed to 4 h of 1% O₂ (hypoxia: Hx) with the indicated concentrations of midazolam. In each case, controls were exposed to 4 h of 20% O₂. (A) Glucose transporter 1 (GLUT1), (B) lactate dehydrogenase A (LDHA) and (C) vascular endothelial growth factor (VEGF) mRNA were assayed with real-time qRT-PCR. Data are shown as mean $\pm$ SD ($n=3$). The expression levels of 18S mRNA were used as an internal standard to normalize the expression levels of GLUT1, LDHA and VEGF. The relative quantifications to the mean of control mice are shown. * $P < 0.01$ vs. control; N.S., not significant.

networks (Milano and Collomp, 2005). Previous studies have shown that in the absence of EPO or the EPO receptor, increased neuronal apoptosis was detected in mice just before the mice died from severe anemia *in utero* (Yu et al., 2001; Yu et al., 2002). We investigated the effects of midazolam on EPO in fetal mice brains. The fetus is physiologically hypoxic because the blood in the umbilical vein going to the fetus is 80–90% saturated with PaO₂ 32–35 mmHg (Puente et al., 2014). Thus, in the present study, midazolam was administered to pregnant mice under normoxic and hyperoxic conditions. The results from this experiment showed that midazolam suppressed the expression of EPO in the fetal brains; however, exposing the pregnant mice to hyperoxic conditions did not produce a suppressive effect on EPO in the fetal brains. Theoretically, maternal exposure to high concentrations of

oxygen should ameliorate the hypoxic condition of the fetus and attenuate the production of EPO. However, one potential explanation that a suppressive effect did not occur may be because of the arteriovenous mixing of the blood of the fetus through the foramen ovale. The saturation of the blood for the fetal brain may not have been significantly elevated; thus, the attenuation for the CNS-derived EPO did not occur. In the present study, the arterial oxygen concentration was not examined in the mice fetus because it was technically difficult. Additional studies are required to investigate the effect of hyperoxia in other animals. Because CNS-derived EPO is essential for neurogenesis, the suppressive effect of midazolam on CNS-derived EPO may significantly impact the developing brain. A number of reports have shown the toxicity of anesthetics including midazolam against neuronal development

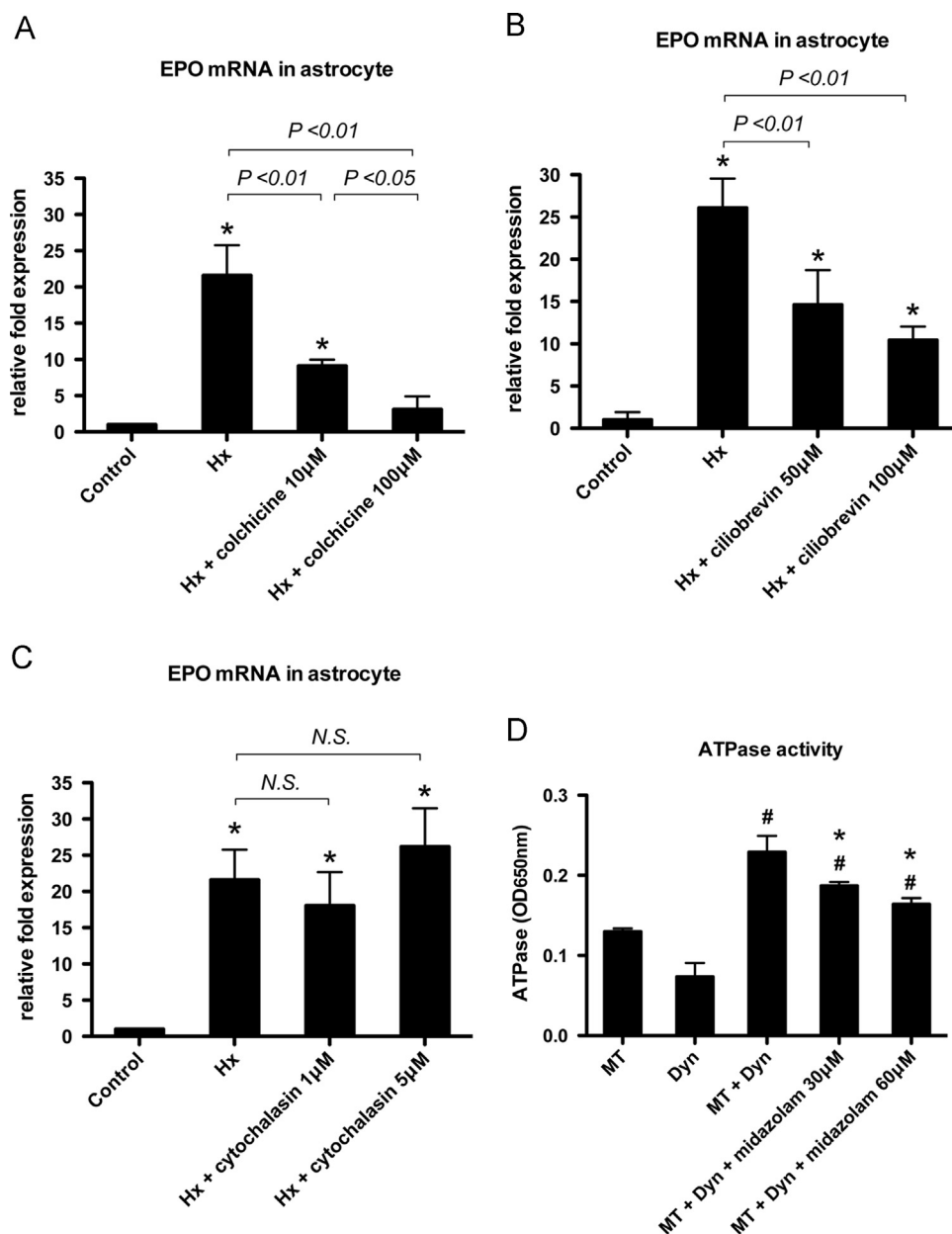


Fig. 6. Mechanism of the midazolam-induced suppression against nuclear accumulation of HIF- α proteins in astrocytes. Primary astrocyte cell cultures were exposed to 4 h of 1% O₂ (hypoxia: Hx) with the indicated concentrations of (A) colchicine, (B) ciliobrevin and (C) cytochalasin. In each case, controls were exposed to 4 h of 20% O₂. EPO mRNA was measured with real-time qRT-PCR. Data are shown as mean $\pm$ SD ($n=3$). The expression levels of 18 S mRNA were used as an internal standard to normalize the expression levels of EPO. The relative quantifications to the mean of control are shown. * $P < 0.01$ vs. control; N.S., not significant. (D) ATPase activity of the cytoplasmic dynein purified from porcine brain was measured by monitoring real-time free phosphate generation. In this assay, the relative ATPase activity correlates with an absorbance of 650 nm. Data are presented as the mean of three independent experiments. MT, microtubule; Dyn, dynein. # $P < 0.01$ vs. MT or Dyn; * $P < 0.01$ vs. MT + Dyn.

(Jevtovic-Todorovic et al., 2003; Sanders et al., 2013). Future studies need to examine the effects of anesthetics on EPO from the developing brain.

To further investigate the mechanism of midazolam-induced EPO suppression under hypoxic conditions, we investigated HIF protein levels in primary cultured astrocytes. EPO is a HIF target gene in CNS (Stockmann and Fandrey, 2006). HIF-1 α expression is ubiquitous, and HIF-2 α expression is tissue-specific (Ema et al., 1997; Wang et al., 1995). HIF-2 α is expressed in astrocytes and endothelial cells in CNS (Ema et al., 1997) and is considered to be the predominant regulator of hypoxia-induced EPO expression in astrocytes (Chavez et al., 2006), with HIF-1 α contributing to a lesser extent (Wang et al., 1995). In the present study, we found that the nuclear accumulation of HIF-1 α and HIF-2 α proteins under hypoxic conditions was significantly suppressed with

midazolam. On the other hand, total HIF-1 α and HIF-2 α levels remained almost unchanged, suggesting that nuclear translocation of HIF- α proteins is inhibited by midazolam. According to recent reports, the nuclear translocation of certain transcription factors, including HIF, p53, and androgen receptors, depends on a microtubule-based transport system (Carbonaro et al., 2012; Giannakou et al., 2002; Thadani-Mulero et al., 2012). These transcription factors are transported into the nucleus by the movement of dynein along the cytoskeletal microtubules (Thadani-Mulero et al., 2012). In this study, colchicines and ciliobrevin (Firestone et al., 2012) suppressed the up-regulation of EPO under hypoxic conditions in primary cultured astrocytes. These results suggest that HIF- α proteins are translocated into the nucleus by a microtubule-based transport system in astrocytes. In the present study, we showed that midazolam significantly inhibited the ATPase activity

of cytoplasmic dynein. This result suggests that midazolam suppresses EPO expression by restricting the microtubule-based transport of HIF proteins by dynein. Many anesthetics have this suppressive effect on EPO expression; thus, these drugs may have a common mechanism of action in which the ATPase activity of dynein is directly inhibited. On the other hand, in the present study, inhibitory effect of midazolam on the dynein ATPase activity was not so distinctive in comparison with that of HIF nuclear accumulation and EPO induction. A microtubule-based transport system is influenced by various factors, for example, microtubule post-translational modifications (PTMs) or microtubule-associated proteins (MAPs) (Franker and Hoogenraad, 2013). The effect of anesthetics including midazolam on such factors is not well studied, although isoflurane has been shown to bind and affect microtubule cytoskeleton (Craddock et al., 2012). Cytoplasmic dynein transports organelles and assembles centrosomes, which are essential functions for cell survival (Schiavo et al., 2013; Vallee et al., 2012). Thus, future precise studies are required to investigate the effects of anesthetics on the microtubule-based transport system.

In the present *in vitro* study, we found that midazolam strongly reduces the increases in EPO mRNA expression and EPO concentration in the culture medium. In contrast, a higher concentration of midazolam is required to reduce the increases in the expression of genes regulated by HIF other than EPO: GLUT1 mRNA, LDHA mRNA, and VEGF mRNA. A possible explanation for this discrepancy is that inductions of those genes under hypoxic conditions are influenced by factors other than HIF. For example, the induction of VEGF is affected by other transcription factors such as GATA-binding protein 2 (GATA2) and nuclear factor-kappa B (NF- κ B) (La Ferla et al., 2002). LDHA is influenced by the pyruvate kinase muscle isozyme (PKM) 2 (Luo et al., 2011) and GLUT-1 by serum response factor (SRF) (Ebert et al., 1995). Such molecules may have affected the inhibitory effect of midazolam. Further research is required to clarify the detailed mechanism.

5. Conclusions

The present study demonstrated that midazolam suppresses the hypoxia-induced up-regulation of EPO *in vivo* and *in vitro*, most likely through the inhibition of HIF- α protein nuclear translocation. The administration of midazolam also suppresses EPO in fetal mice brains. Because CNS-derived EPO is essential for neurogenesis and neuroprotection, midazolam may significantly impact the clinical treatment of patients, particularly patients with brain hypoxia or patients who are pregnant.

Authors' contributions

T.M. performed most of the experiments and helped writing the manuscript. H.D., S.K., K.T. and H.H. helped in conducting the experiments. T.T. performed some experiment, wrote the manuscript and contributed to the design of the experiments. K.F. supervised the experiments and writing of the manuscript.

Declaration of interest

None declared.

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