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The volatile anesthetic isoflurane differentially suppresses the induction of erythropoietin synthesis elicited by acute anemia and systemic hypoxemia in mice in an hypoxia-inducible factor-2-dependent manner

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ABSTRACT

Erythropoietin (EPO) is a glycoprotein hormone essential for the regulation of erythroid homeostasis. Although EPO production is prominent in the kidney and liver, its production in the central nervous system has also been detected. Tissue hypoxia due to systemic or local hypoxemia and acute anemia due to blood loss occurs frequently during various clinical settings, leading to a high possibility of elevated plasma EPO levels. However, it is largely unknown whether volatile anesthetic agents affect EPO production elicited by acute hypoxia *in vivo*. Male C57BL/6N CrSlc mice were exposed to a hypoxic insult as a result of bleeding-related anemia or hypoxemia while they were under anesthetized using various concentrations of isoflurane. EPO protein concentrations were assessed by enzyme-linked immunosorbent assay and mRNA levels were measured by quantitative real-time reverse transcriptase-polymerase chain reaction. Plasma EPO concentration was induced as early as 3 h following acute anemic and hypoxemic hypoxia and suppressed by clinically relevant doses of isoflurane in a dose-dependent manner. Anemic hypoxia induced EPO mRNA and protein synthesis in the kidney. In the kidney, isoflurane inhibited EPO induction caused by anemia but not that caused by hypoxemia. On the other hand, in the brain hypoxemia-induced EPO production was suppressed by isoflurane. Finally, qRT-PCR studies demonstrate that isoflurane differentially inhibit HIF-1α and HIF-2α mRNA expression in brain and kidney, indicating the involvement of HIF-2 in the hypoxia-induced EPO expression and inhibition of the induction by isoflurane.

1. Introduction

Erythropoietin (EPO) is a glycoprotein hormone, essential for the regulation of erythroid homeostasis (Adamson, 1991; Koury, 2005; Semenza et al., 1989). Although EPO production is detectable in almost all the cells of the body, it is most prominent in the kidney and liver (Fandrey and Bunn, 1993; Huang and Bunn, 1995). EPO production in the central nervous system, including the brain and spinal cord, has been identified (Brines and Cerami, 2005; Noguchi et al., 2007). The physiology of cerebral EPO is incompletely understood, although some studies have shown it to play roles in neural development and neuroprotection in addition to erythroid system development. Steady-state production of EPO is necessary to maintain the physiological daily renewal of red blood cells. EPO synthesis is highly induced by anemia, decreased ambient oxygen tension, increased oxygen affinity for hemoglobin, and other stimuli that decrease delivery of oxygen to the tissues. EPO synthesis is strictly regulated at the transcriptional level. The EPO gene is mainly activated by hypoxia-inducible factors (HIFs) including HIF-1 and HIF-2, which bind to the hypoxia-sensitive region of the EPO gene to activate it (Fandrey, 2004; Jelkmann, 2011; Maxwell and Ratcliffe, 1998). In addition, tonic inhibitory regulation by a family of GATA transcription factors has been identified (Imagawa et al., 1989, 1997; Obara et al., 2008).

We previously showed that induction of EPO expression under hypoxemic hypoxic conditions was suppressed by general anesthetic agents such as isoflurane in a concentration- and time-dependent manner in the mouse brain through suppression of HIF-2 activity (Tanaka et al., 2011). Clinically, tissue hypoxia due to systemic or local hypoxemia and acute anemia due to blood loss occurs frequently in the perioperative period, which increases the

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possibility of elevated plasma EPO levels. However, it is largely unknown when and where EPO induction occurs in response to hypoxic insults *in vivo* and whether volatile anesthetic agents affect EPO production elicited by such acute hypoxia *in vivo*.

In this study, we demonstrate that bleeding-related anemia and acute systemic hypoxemia differentially induce expression of EPO protein and mRNA synthesis in the brain and kidney, and that isoflurane differentially suppresses the inductions.

2. Materials and methods

2.1. Animals

Animal protocols were approved by the Animal Research Committee of Kyoto University (med-kyt #12155, Kyoto University, Japan), and all experiments were conducted in accordance with the institutional and National Institute of Health guidelines for the care and use of laboratory animals (Kai et al., 2012; Tanaka et al., 2011). All procedures were performed on 9-week-old, male, C57BL/6N CrSlc mice purchased from Japan SLC Inc., Shizuoka, Japan. Food and water were provided *ad libitum*, and the mice were maintained under controlled environmental conditions (24 h, 12-h light/dark cycle).

2.2. Drugs and chemicals

Isoflurane was obtained from Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan. Oxygen (Taiyo Nippon Sanao, Wakayama, Japan), and nitrogen (Taiyo Nippon Sanso) were also used (Tanaka et al., 2011).

2.3. Induction of anemia

To expose mice to hypoxic conditions simulating bleeding-related anemia, 0.2 ml of blood was removed via the retro-orbital venous plexus (Obara et al., 2008; Weidemann et al., 2009). Hemoglobin concentrations and hematocrit values were measured to confirm the establishment of anemia.

2.4. Exposure to isoflurane and hypoxemic treatment

Isoflurane treatment was described previously (Tanaka et al., 2011). Briefly, mice were placed in a polypropylene chamber, to which oxygen and nitrogen mixed gas, with or without the volatile anesthetic isoflurane, was delivered at a flow rate of 3 l/min using an anesthesia machine (Custom50; Aika, Tokyo, Japan). Concentrations of oxygen, carbon dioxide, and isoflurane were monitored continuously using an infrared analyzer (Capnomac Ultima; Datex-Ohmeda, Helsinki, Finland).

The hypoxemic hypoxia protocol was described previously (Tanaka et al., 2011). Mice were exposed to and maintained in a 10% oxygen hypoxic environment for predetermined durations. Pulse rate and arterial blood pressure were noninvasively measured using the tail-cuff method immediately after completion of the hypoxic exposure using an MK-2000ST (Muromachi Kikai Co., Ltd, Tokyo, Japan). Arterial oxygen saturation (SpO_2) was monitored by the pulse oximetry MouseOx pulse oximeter (Starr Life Sciences, Oakmont, PA). At the end of the study period, mice were euthanized by cervical dislocation and the kidneys, brain, and liver rapidly removed, frozen in liquid nitrogen, and stored at $-80\,^{\circ}C$ for subsequent analysis.

2.5. Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

RNA was isolated from the frontal lobe of the brain, the whole kidney, and a liver lobe using a FastPure™ RNA Kit (Takara Bio, Inc., Shiga, Japan) (Kai et al., 2012; Tanaka et al., 2011). First-strand synthesis and real-time quantitative PCR was performed using One Step SYBR™ (Takara Bio) and an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA). PCR primers were purchased from Qiagen (Valencia, CA, USA) (Kai et al., 2012; Tanaka et al., 2011). Fold changes in expression of each target mRNA were calculated relative to 18S mRNA (Kai et al., 2012; Tanaka et al., 2011).

2.6. Plasma EPO evaluation

Blood was withdrawn from the heart into a heparinized syringe. Plasma was separated and assayed by an ELISA kit (R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions (Tanaka et al., 2011). Values of optical density were measured spectrophotometrically (Microplate Reader 680 XR; Bio-Rad) at 450 nm (correction wavelength set at 570 nm). The standard curve was made with PrismTM software. In each experiment, all samples and standards were measured in duplicate and the amount of EPO expressed in pg/mL.

2.7. Tissue EPO determination

Samples were prepared according to the method described previously (Tanaka et al., 2011). Briefly, kidney, brain, and liver were homogenized in phosphate-buffered saline, centrifuged for 10 min at 5000*g* at 4 °C, and immediately frozen at $-20\,^{\circ}C$. After two freeze–thaw cycles to break up the cell membranes, tissue homogenates were assayed with an ELISA kit (R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions. The results were expressed as the ratio of the quantity of EPO (in pg) to the quantity of total protein (in mg) in the organs. The total protein concentration was determined by the modified Bradford assay (Nakalai Tesque, Inc., NC, Kyoto, Japan) using bovine serum albumin as a standard.

2.8. Statistical analyses

Data are presented as means ± S.D. Statistical significance was assessed by a Mann–Whitney *U*-test for between group comparisons, and by a Kruskal–Wallis *H*-test, followed by a Mann–Whitney *U*-test with Bonferroni Correction for multiple comparisons using Prism version 5. $P < 0.05$ was considered statistically significant (Tanaka et al., 2011).

3. Results

3.1. Acute anemia and systemic hypoxemia induce an increase in plasma EPO concentrations

To examine the plasma EPO, 9-week-old mice were bled by taking 0.2 ml of blood from retro-orbital plexus under room air conditions at 3, 5, and 10 h before analysis or exposed to 10% O_2 conditions for 3, 5, and 10 h. Plasma EPO levels were measured using ELISA (Fig. 1). The average hemoglobin concentration in acutely anemic mice decreased from 15.1 g/dl to 11.5 g/dl, and the mean hematocrit value decreased from 44.4% to 33.8% after bleeding.

Table 1 details the physiological variables tracked before and during the treatments.