The intravenous anesthetic propofol inhibits hypoxia-inducible factor 1 activity in an oxygen tension-dependent manner

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**Abstract   Hypoxia elicits a wide range of responses that occur at different organizational levels in the body. Hypoxia is not only a signal for energy conservation and metabolic change, but triggers expression of a select set of genes. The transcription factor hypoxia-inducible factor 1 (HIF-1) is now appreciated to be a master factor of the gene induction. Although knowledge on molecular mechanisms of HIF-1 activation in response to hypoxia is accumulating, the molecular mechanism of maintenance of HIF-1 activity under normoxic conditions remains to be elucidated. We demonstrate that the intravenous anesthetic propofol reversibly inhibits HIF-1 activity and the gene expression mediated by HIF-1 by blocking the synthesis of the HIF-1α subunit under 20% or 5% O_2 conditions, but not under 1% O_2 conditions.
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1. Introduction

Adaptation to hypoxia and maintenance of O_2 homeostasis involve a wide range of cellular and systemic responses that occur at different organizational levels in the body [1]. Hypoxia induces expression of a select set of genes encoding glycolytic enzymes and glucose transporters that generate ATP under anaerobic conditions [2,3]. The transcription factor hypoxia-inducible factor 1 (HIF-1) plays an essential role in the maintenance of O_2 homeostasis [3]. HIF-1 is a heterodimer, consisting of HIF-1α and HIF-1β subunits, which bind to specific regulatory sequences known as hypoxia response elements (HREs). The biological activity is determined by the expression and activity of the HIF-1α subunit [4]. HIF-1α protein expression level is determined by the balance between protein synthesis and degradation. The mechanisms regulating HIF-1α protein expression and transcriptional activity have been most extensively analyzed. The von Hippel–Lindau tu-

mor-suppressor protein (VHL) has been identified as the HIF-1α-binding component of a ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation in non-hypoxic cells. Under hypoxic conditions, the hydroxylation of specific proline and asparagine residues in HIF-1α is inhibited due to substrate (O_2) limitation, resulting in HIF-1α protein stabilization and transcriptional activation [5]. The iron chelator desferrioxamine (DFX) inhibits the prolyl (prolyl hydroxylase domain containing protein [PHD] 1–3) and asparaginyl (factor inhibiting HIF-1 [FIH-1]) hydroxylases, which contain Fe^{2+} at their catalytic sites, causing HIF-1α stabilization and transactivation under normoxic conditions [5]. On the other hand, we have reported that certain growth factors such as IGF-1 [6], prostaglandin E_2 [7], and the nitric oxide donor NOC18 [8] increase the rate of HIF-1α protein synthesis rather than increasing the stability of HIF-1. Activated HIF-1 induces expression of its target genes, which play key roles in glycolytic energy metabolism, erythropoiesis and angiogenesis even under non-hypoxic conditions as well as hypoxic conditions [9]. However, few study have investigated the inhibitory regulation of HIF-1 activity under non-hypoxic conditions.

We previously reported that the volatile anesthetic halothane strongly inhibits the hypoxia-induced HIF-1 activation in the established cell line Hep3B [10]. In this study we focus on the intravenous anesthetic propofol. So far there has been no report about the effect of propofol on the hypoxia-induced gene responses mediated by HIF-1. In this study we demonstrate that propofol inhibits HIF-1 activity by suppressing HIF-1α protein synthesis and that the inhibition is dependent on the cellular oxygen tension.

2. Materials and methods

2.1. Cell culture and reagents
Hep3B cells were maintained in minimum essential medium (MEM) with Earle's salts supplemented with 10% fetal bovine serum (FBS), essential amino acids, pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin [8,11]. HEK293 cells was maintained in Dulbecco's MEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin [8,11]. Human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo (Osaka, Japan). The intravenous anesthetic 2,6-Diisopropylphenol (Propofol) and its inactive analogue 2,4-Diisopropylphenol as an anesthetic, DMSO as solvent,

and the iron chelator desferrioxamine (DFX) were obtained from Sigma (St. Louis, MO). The inhibitor of protein synthesis cycloheximide (CHX) and the cell-permeable proteasome inhibitor MG132 were from Calbiochem (San Diego, CA). The antioxidants N-acetyl-cysteine (NAC) and dithiothreitol (DTT) were from Calbiochem (San Diego, CA). Ascorbate and α-tocopherol were from Sigma (St. Louis, MO).

2.2. Plasmid constructs

Reporter plasmid p2.1, which contains a 68-bp HRE from the human enolase 1 (ENO1) gene, an SV40 promoter, and luciferase coding sequences, was described previously [8,11,12]. The *Renilla* luciferase expression plasmid pRL-SV40 was from Promega (Madison, WI).

2.3. Hypoxic treatment

Cells were maintained in a multi-gas incubator (APMW-36, Astec, Japan) and were exposed to hypoxia (1% O_2–5% CO_2–94% N_2 or 5% O_2–5% CO_2–90% N_2) at 37 °C [8,11].

2.4. Immunoblot assays

Whole cell lysates were prepared using ice-cold lysis buffer (0.1% SDS, 1% NP-40, 5 mM EDTA, 150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 2 mM DTT, 1 mM sodium orthovanadate, and complete protease inhibitor [Roche Applied Science, Tokyo Japan]) following a protocol described previously [8,11]. 100-µg aliquots were fractionated by 7.5% SDS–PAGE and subjected to immunoblot assay using mouse monoclonal antibody against HIF-1α (BD Biosciences, San Jose, CA) or HIF-1β (H1β234; Novus Biologicals, Littleton, CO) at 1:1000 dilution and HRP-conjugated mouse monoclonal antibodies for mouse IgG (Amersham Bioscience, Piscataway, NJ, 1:1000 dilution). Anti-ERK3 antibody was from SantaCruz and used at 1:1000 dilution (San Diego, CA). Chemiluminescent signal was developed using ECL reagent (Amersham Biosciences).

2.5. RT-PCR

The RT-PCR protocol is described elsewhere [8]. Cells were harvested, and RNA was isolated with TRIzol (Invitrogen). 1 µg of total RNA was subjected to first strand cDNA synthesis using random hexamers (SuperScript II RT kit, Invitrogen). cDNAs were amplified with TaqGold polymerase (Roche, Manheim, Germany) in a thermal cycler with the specific primers (sequences of the primers can be provided by request). For each primer pair, PCR was optimized for cycle number to obtain linearity between the amount of input RT product and output PCR product. Thermocycling conditions were 30 s at 94 °C, 60 s at 57 °C, and 30 s at 72 °C for 20 (lactate dehydrogenase [LDHA]), 25 (vascular endothelial growth factor [VEGF]), 30 (PHD-1), 30 (PHD-2), 30 (PHD-3), 30 (FIH-1), 25 (HIF-1α), and 20 (18S rRNA) cycles preceded by 10 min at 94 °C. PCR products were fractionated by 1.5% SeaKem GTG Agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV. Quantification of amplified mRNA was done by densitometry assisted by the image analysis software NIH Image (version 1.62).

2.6. Reporter gene assay

Reporter assays were performed in Hep3B cells [8,11]. 5×10^4 cells were plated per well and incubated for an overnight, and were subsequently transfected 200 ng of reporter gene plasmid p2.1 and 50 ng of pRL-SV40 pre-mixed with Fugene 6 transfection reagent (Roche). Cells were treated with the reagents in 6 h, and incubated with or without propofol under normoxic or hypoxic conditions for another 18 h. The cells were harvested and the luciferase activity of each well was measured in the same dosage of cell lysate with the use of Dual-Luciferase Reporter Assay System (Promega). For each experiment, at least two independent transfections were performed in triplicate and representative data are shown.

2.7. In vitro HIF-1α-VHL interaction assay

Glutathione S-transferase (GST)-HIF-1α (429–608) fusion protein was expressed in *Escherichia coli* as described. Biotinylated methionine-labeled proteins were generated in reticulocyte lysates using the TNT T7 coupled transcription/translation system and Transcend Biotinylated tRNA™ (Promega). 25-µg aliquots of HEK293 cell lysate were preincubated with propofol or DFX for 30 min at 30 °C, 5 µg of GST-HIF-1α (429–608) was added and incubated for 30 min at 30 °C. A 5-µl aliquot of in vitro-translated biotinylated VHL protein was mixed with 5 µg of GST fusion protein in a final volume of 200 µl of

HEB buffer [20 mM Tris (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol] and incubated for 30 min at 4 °C with rotation followed by addition of 10 µl of glutathione-Sepharose 4B beads (Amersham Bioscience) and incubation at 4 °C for 1 h. The beads were pelleted, washed 3 times in NETN buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, and 0.5% NP-40], pelleted again, resuspended in Laemmli sample buffer, and analyzed by SDS–PAGE. Proteins were transferred to PVDF membrane and visualized using streptavidin-labeled horseradish peroxidase and ECL reagent (Amersham Bioscience).

3. Results

3.1. Effect of propofol on HIF-1α and HIF-1β protein expression and its dependency on O_2 concentration

To examine the effect of propofol on HIF-1 activity, Hep3B cells were treated with or without propofol under non-hypoxic (20% O_2) or hypoxic (1% O_2) conditions. HIF-1α protein levels were low under non-hypoxic conditions, and increased markedly in response to hypoxic conditions (Fig. 1A, upper; lanes 1 and 3). 100 µM propofol inhibited the expression of HIF-1α under non-hypoxic conditions (lanes 1 and 2). In contrast, hypoxic induction of HIF-1α protein accumulation was not suppressed by propofol treatment (lanes 3 and 4). Expression of HIF-1β was not affected by exposure to hypoxia or propofol (Fig. 1A, lower). The suppressive effect of propofol on the expression of HIF-1α under non-hypoxic conditions was dependent on concentrations of propofol $\geqslant 10$ µM (Fig. 1B).
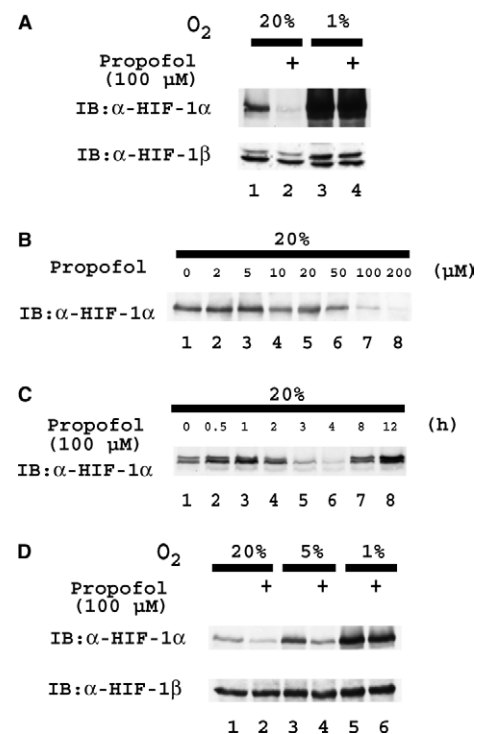

Fig. 1. Propofol inhibits HIF-1 activity in Hep3B cells. Hep3B cells were exposed to 20% or 1% O_2 with or without 100 µM propofol for 4 h and harvested for immunoblot assays using anti-HIF-1α and -HIF-1β Abs (A). Studies on dose-dependency (B) and time-course (C) were performed in indicated conditions. In (D), Hep3B cells were exposed to 20%, 5%, or 1% O_2 with or without 100 µM propofol.

The effect of propofol peaked at 4 h and was lost by 8 h after addition (Fig. 1C).

To investigate if the effect of propofol on HIF-1α protein accumulation is O_2-concentration-dependent or not, Hep3B cells were exposed to 20%, 5%, or 1% O_2 for 4 h with or without 100 μM of propofol. Induction of HIF-1α protein was inversely correlated with O_2 concentration (Fig. 1D, lanes 1, 3, and 5). Propofol suppressed HIF-1α protein expression in cells exposed to 20% or 5% O_2 (lanes 2 and 4). In contrast, induction of HIF-1α protein expression at 1% O_2 was not significantly suppressed by propofol (lane 6).

3.2. Effects of propofol are not cell-specific

In addition to in Hep3B cells, propofol suppressed the basal expression of HIF-1α under normoxic conditions in HEK293 cells (Fig. 2A) and HUVECs (Fig. 2B). HIF-1α accumulation in cells exposed to 1% O_2 was not affected in either HEK293 cells or HUVECs.

3.3. Effect of propofol on HIF-1-dependent gene expression

We investigated the effect of propofol on hypoxia-induced HIF-1-mediated gene expression. The mRNA expression of genes was assayed using RT-PCR technique. Propofol suppressed the basal expression of mRNA of LDHA, VEGF, PHD-3 and FIH-1 under non-hypoxic conditions but not under hypoxic conditions, data which are consistent with the effect of propofol on HIF-1α protein levels (Fig. 3A). Expression of PHD-1, PHD-2 and HIF-1α mRNA was not affected by either propofol or hypoxia.

The effect of propofol on HIF-1 activity was also investigated in Hep3B cells using an HRE-luciferase reporter construct. Twenty μM of propofol inhibited the hypoxia-induced HRE-dependent gene expression only marginally whereas 100 μM of propofol inhibited reporter gene expression by about 35% (Fig. 3B).

3.4. Effect of antioxidants on HIF-1α protein expression

Propofol is a phenol and reported to serve as an antioxidant [13]. To approach the molecular mechanism of propofol action, we decided to use other well-known antioxidants (Fig. 4A). HIF-1α expression was suppressed by propofol (lane
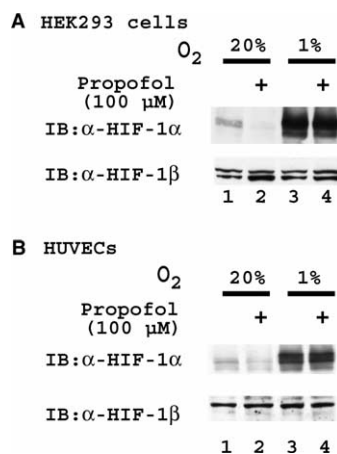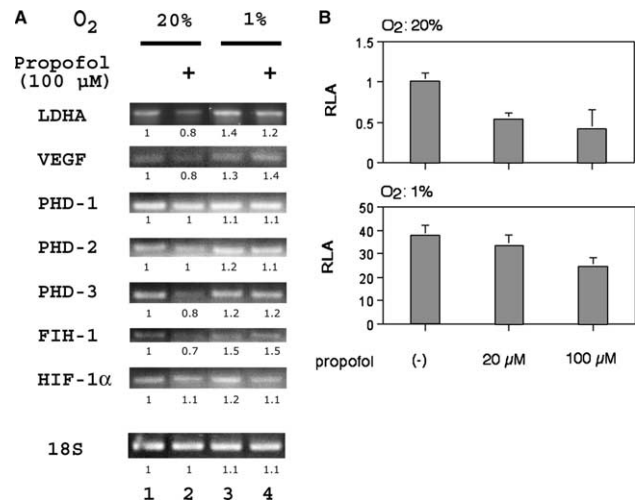

Fig. 3. Effect of propofol on the expression of HIF-1-dependent genes or related genes. (A) Hep3B cells were exposed to 20% or 1% O_2 with or without 100 μM propofol for 24 h and total RNA was isolated. Expressions mRNA of LDHA, VEGF, PHD-1-3, FIH-1 and HIF-1α and 18s were analyzed by RT-PCR using specific primer pairs. Quantification of amplified mRNA was done by densitometry assisted by the image analysis software NIH Image (version 1.62) and indicated as fold induction to each lane 1. (B) Hep3B were transfected with p2.1 reporter. After 6 h incubation, cells were treated with 20 or 100 μM propofol under 20% (upper) or 1% O_2 (lower) for 18 h and harvested for luciferase assays. The relative luciferase activity (RLA) was based on the value of non-hypoxic untreated cells. Results were shown represent mean ± S.D. of six independent transfections.
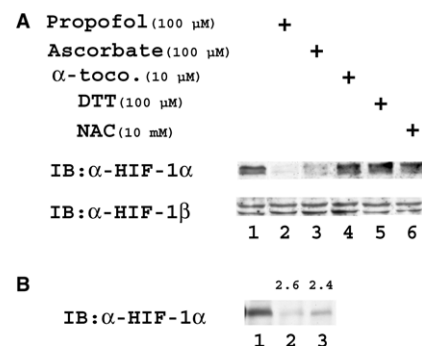

Fig. 4. Comparison of propofol and other antioxidants. (A) Hep3B cells were exposed to propofol (100 μM), ascorbate (100 μM), α-tocopherol (α-toco.) (10 μM), DTT (100 μM), and NAC (10 mM) for 4 h under 20% O_2 conditions. (B) Hep3B cells were exposed to 2,6-Diisopropylphenol (propofol) and 2,4-Diisopropylphenol for 4 h under 20% O_2 conditions. Lysates were prepared and were subjected to Western blot using anti-HIF-1α Ab.

2). In contrast, neither α-tocopherol (lane 4), DTT (lane 5), nor NAC (lane 6) suppressed the expression of HIF-1α. In contrast, ascorbate, which is a potent activator of the HIF-1α prolyl hydroxylases, suppressed the expression (lane 3). 2,4-Diisopropylphenol is an isomer of propofol (2,6-diisopropylphenol) and does not have a potency to bind to GABA receptor of neuronal cells. Both 2,6-diisopropylphenol (propofol) (Fig. 4B, lane 2) and 2,4-diisopropylphenol (lane 3) suppress the expression of HIF-1α under 20% conditions, suggesting that the effect of propofol is not explained via GABA receptor binding.

Fig. 2. The effects of propofol on HIF-1 in HEK293 cells and HUVECs. HEK293 cells (A) and HUVECs (B) were exposed to 20% or 1% O_2 with or without 100 μM propofol for 4 h and harvested for immunoblot assays using anti-HIF-1α and -HIF-1β Abs.

3.5. Effect of propofol on the degradation and synthesis of HIF-1α

HIF-1α levels are determined by the balance between synthesis and degradation [5]. The O_2-dependent degradation of HIF-1α is governed by the activity of prolyl hydroxylases of HIF-1α and subsequent ubiquitination of HIF-1α by a ubiquitin ligation system that includes VHL as an essential component [5]. Interaction between HIF-1α and VHL is governed by hydroxylation status of HIF-1α. We investigated the effects of propofol on the prolyl hydroxylation of HIF-1α using an in vitro assay system. Propofol did not affect the prolyl hydroxylation of HIF-1α (Fig. 5A, lane 3). In contrast, DFX inhibited hydroxylation (lane 4). We next examined the effect of 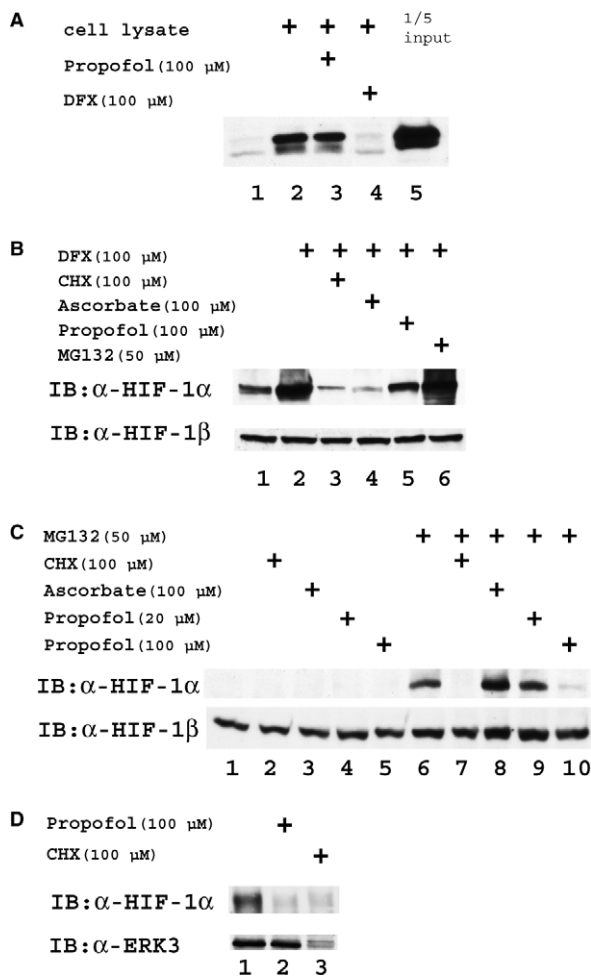propofol on HIF-1α accumulation induced by the iron chelator DFX. Propofol decreased the expression of HIF-1α in cells exposed to 100 μm DFX (Fig. 5B, lane 5). Exposure of cells to CHX (lane 3) or ascorbate (lane 4) had inhibitory effect on HIF-1α levels, although the mechanism of the inhibition differs. CHX decreased HIF-1α protein by inhibition of protein translation whereas ascorbate, which is a cofactor for the prolyl hydroxylases, stimulated HIF-1α protein degradation. Exposure of cells to MG132, a proteasome inhibitor, causes cells to accumulate HIF-1α protein (lane 6). Hep3B cells were treated with propofol or ascorbate in the presence of MG132 (Fig. 5C). Ascorbate did not suppress the accumulation of HIF-1α under MG132 (lane 8). Propofol (lanes 9 and 10) as well as CHX (lane 7) inhibited the accumulation of HIF-1α induced by MG132 treatment in a dose dependent manner. Finally the specificity of propofol as an inhibitor of protein synthesis was investigated (Fig. 5D). ERK3 is a protein with rapid turnover and mainly degradated by ubiquitin–proteasome system [14]. Notably, propofol selectively suppresses HIF-1α protein synthesis (lane 2). In contrast, CHX, which is a general translational inhibitor, suppresses both HIF-1α and ERK3 synthesis (lane 3).

Fig. 5. Effect of propofol on the degradation or synthesis of HIF-1α. (A) GST-HIF-1α (429–608) fusion protein was incubated with in vitro translated VHL in the presence of PBS or cell lysates untreated or treated with the indicated reagents (lanes 1–4). Glutathione-Sepharose beads were used to capture GST-HIF-1α (429–608) and the presence of bound VHL was determined by PAGE. One-fifth of the input VHL protein was also analyzed (lane 5). (B) Hep3B cells were treated with 100 μM CHX, ascorbate, propofol, and 50 μM MG132 under coexistence with 100 μM DFX for 4 h in 20% O_2. (C) Hep3B cells were treated with 100 μM CHX, 100 μM ascorbate, 20 μM propofol, or 100 μM propofol coexistent with or without 50 μM MG132. (D) Hep3B cells were treated with 100 μM propofol or 100 μM CHX. Lysate were analyzed by Western blot using anti-HIF-1α and ERK3 Abs.

4. Discussion

In this report we have demonstrated a reversible inhibitory effect of propofol on HIF-1 activity. The suppressive effect of propofol on HIF-1α protein expression was significant at 20% and 5% O_2 but not at 1% O_2. Propofol suppressed HIF-1 activity under 20% O_2 assayed by RT-PCR (Fig. 3A) and an HRE-luciferase reporter construct (Fig. 3B).

Several reports indicate that propofol has antioxidant activity related to its phenolic group [13,15] and intracellular redox balance is known to affect HIF-1 activity [16]. But our results of Fig. 4A, especially the result from α-tocopherol, strongly suggested that the antioxidant activity of propofol does not explain the suppressive effect.

O_2-dependent regulation of HIF-1 activity occurs via multiple mechanisms including effects on HIF-1α expression, intracellular localization of the HIF-1α:HIF-1β complex, and transcriptional activity of HIF-1α. Among them, regulations of HIF-1α expression level and transcriptional activity are the most crucial steps. The expression of HIF-1α is determined by a balance between degradation and synthesis. In this study, we demonstrate that propofol does not inhibit the interaction between HIF-1α and VHL and that propofol does not suppress hypoxia-induced HIF-1α accumulation significantly. The data from Fig. 5C indicate that propofol suppress the accumulation of HIF-1α as well as CHX, a protein translation inhibitor, even after inhibition of proteasome system. Taken together with data that propofol does not influence HIF-1α mRNA expression (Fig. 3A), our results indicate that propofol does not affect the HIF-1α-hydroxylase activity but suppresses the translation of HIF-1α mRNA into protein. Although the details of molecular process of HIF-1α synthesis are still to be elucidated, mitogen activation protein kinase (MAPK) and PI3 kinase activity play significant roles in determining the translational rate of HIF-1α from mRNA [8]. In fact, propofol is reported to suppress MAPK activity [17]. The inhibitory effect of propofol on HIF-1α synthesis is specific because propofol did not affect the protein expression

of ERK3, which is degradated rapidly by a ubiquitin–proteasome system (Fig. 5D). In contrast to results shown at the protein level, propofol seems to have an effect on HIF-1 activity judged from luciferase assay (Fig. 3C) in normoxia and in hypoxia. We have experimental data that propofol suppresses the GAL4-HIF-1α-transactivation domain-dependent gene expression under both normoxia and hypoxia (data not shown). The suppression of MAPK, which plays an critical role in regulation of the HIF-1α-transactivity, may contribute to the inhibition. The evidence at least partially explain the discrepancy between the protein expression and the activation.

A notable finding in this study is that the effect of propofol on HIF-1α expression is dependent on O_2 tension. As shown in Fig. 1D, under 20% and 5% O_2 conditions, the inhibitory effect is prominent but under 1% O_2 condition, the effect is not barely observed. Lang et al. [18] reported the presence of IRES in the HIF-1α 5′UTR promoter region. They also indicated that IRES activity is not affected by hypoxic conditions that caused a reduction in cap-dependent translation, and IRES activity was less affected by serum-starvation than was cap-dependent translation. Propofol may affect IRES- and cap-dependent translation differentially.

Propofol is reported to inhibit lactate production [19,20]. On the other hand, a report describes that propofol suppresses macrophage functions and ATP synthesis [21]. In fact, we also observed that propofol decreased cellular lactate and increased cellular pyruvate (manuscript in preparation), and furthermore decreased LDHA mRNA expression (Fig. 3A). The conversion from pyruvate to lactate is an important reaction for supplying NAD^+ to promote glycolytic pathway. Propofol may decrease energy supply in the cells by the suppression of glycolytic pathway, secondary to decreased HIF-1 activity. Inhibitory effects on HIF-1α protein synthesis and on HIF-1 target gene expression were observed at a clinically relevant dose of propofol.

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