Reversible inhibition of hypoxia-inducible factor 1 activation by exposure of hypoxic cells to the volatile anesthetic halothane

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Abstract Volatile anesthetics modulate a variety of physiological and pathophysiological responses including hypoxic responses. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that mediates cellular and systemic homeostatic responses to reduced O_2 availability in mammals, including erythropoiesis, angiogenesis, and glycolysis. We demonstrate for the first time that the volatile anesthetic halothane blocks HIF-1 activity and downstream target gene expressions induced by hypoxia in the human hepatoma-derived cell line, Hep3B. Halothane reversibly blocks hypoxia-induced HIF-1α protein accumulation and transcriptional activity at clinically relevant doses. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Hypoxia-inducible factor 1; Volatile anesthetic; Halothane; Gene expression

1. Introduction

Volatile anesthetics mediate a variety of biological effects, most notably hypnotic effects. In addition to their hypnotic effects on the central nervous system, volatile anesthetics affect the cardiovascular and immune systems by modulating vascular tone, cardiac contraction, and cytokine production. A large number of studies have demonstrated that volatile anesthetics directly affect ligand-gated ion channels, including glutamate receptors, nicotinic acetylcholine receptors and γ-aminobutyric acid A receptors, as well as voltage-gated ion channels, including Na^+, K^+, and Ca^{2+} channels. In addition to plasma membrane ion channels intracellular proteins such as protein kinase C have been shown to be target molecules of anesthetics [1].

Hypoxic stress induces a series of adaptive responses that include alterations of metabolism and gene expression [2]. When availability of oxygen is limited, expression of genes encoding components of the electron transport chain is repressed, while transcription of genes encoding enzymes of the glycolytic pathway is activated. Levels of erythropoietin, the primary growth factor for erythroid progenitor cells and, therefore, a major physiologic regulator of O_2 supply in mammals, increase several hundredfold in rodent liver and kidney in response to hypoxia or anemia. The genes, including genes encoding vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase, are also inducibly expressed in response to hypoxia. Multiple hypoxic responses requiring gene expression are under the control of the transcriptional activator hypoxia-inducible factor 1 (HIF-1) which is a central component in the hypoxic response pathway [3,4].

HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β subunits [3]. To activate transcription of target genes, HIF-1α dimerizes with HIF-1β and the heterodimer binds to DNA at sites represented by the consensus sequence 5′-RCGTG-3′. The HIF-1 binding site is present within a hypoxia-responsive element (HRE), a *cis*-acting transcriptional regulatory sequence that can be located within 5′-flanking, 3′-flanking, or intervening sequences of target genes [3]. Targets of HIF-1 include genes whose protein products are involved in angiogenesis, energy metabolism, erythropoiesis, cell proliferation and death, vascular remodeling, and wound healing [5].

Halothane, which is the prototype of modern volatile anesthetics and is still widely used in clinical settings and animal experiments, has been demonstrated to modulate respiratory function and vascular tone during hypoxia. But so far, to the best of our knowledge, there are no studies on the modulation of hypoxia-induced gene regulation by volatile anesthetics. In this study, using an established cell line derived from a human hepatocellular carcinoma, Hep3B, we demonstrate that halothane over a range of clinically relevant doses reversibly suppresses HIF-1 activation.

2. Materials and methods

2.1. Cell culture and reagents

The human hepatocellular carcinoma-derived cell line, Hep3B, was kindly provided by Dr. Fujii-Kuriyama (Tohoku University, Japan) [6]. Cells were maintained in modified Eagle's medium with Earle's salts and 10% fetal bovine serum (Life Technologies). $CoCl_2$ and desferrioxamine (DFX) were obtained from Sigma. Halothane was obtained from Takeda Chemical Industries (Osaka, Japan).

2.2. Plasmid construction

Reporter plasmid p2.1 harboring a 68-bp HRE from the human enolase 1 (*ENO1*) gene inserted upstream of an SV40 promoter and luciferase coding sequences and its HRE mutant, p2.4, have been described previously [7]. Reporter plasmid P11w harboring a 47-bp HRE from the human VEGF gene and its HRE mutant P11m have been described previously [8]. Expression plasmid pGAL4-HIF-

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Abbreviations: HIF-1, hypoxia-inducible factor 1; HRE, hypoxia-responsive element; DFX, desferrioxamine; ENO1, enolase 1; VEGF, vascular endothelial growth factor; TAD, transactivation domain; VHL, von Hippel–Lindau

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1α(531–826) has been described [9]. Reporter construct ptk-GALpx3-LUC containing three copies of GAL4 binding sites in front of a minimal thymidine kinase promoter and pCMV-GAL4-VP16 were kindly provided by the late Dr. Kazuhiko Umesono (Kyoto University, Kyoto, Japan) [10]. The β-galactosidase expression plasmid, pSV40-β-galactosidase, was obtained from Promega.

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) with or without halothane through a vaporizer [6]. The concentrations of O_2 and halothane in the incubator were monitored during treatment with an anesthetic gas monitor (Type 1304, Brüel and Kjær, Nærum, Denmark) that was calibrated before each study with a commercial standard gas (47% O_2, 5.6% CO_2, 47% N_2O, 2.05% sulfur hexafluoride). The anesthetic concentration in the medium was measured by gas chromatography (Hewlett Packard, 5890A, Palo Alto, CA, USA) as described previously [11].

2.4. Immunoblot assays of HIF-1α

Nuclear extracts were prepared following an established protocol [9]. Aliquots were fractionated by SDS–PAGE and subjected to immunoblot assay using protein G-purified mouse monoclonal antibody H1α67 (Novus Biologicals) at 1:1000 dilution [12,13]. Signal was developed using ECL-Plus reagents (Amersham).

2.5. Reporter gene assays

Reporter gene assays were performed following a protocol described previously [10,13–15]. The relative fold induction of luciferase activity was calculated after dividing the luciferase activity by β-galactosidase activity. Each experiment was done at least two times in triplicate and data from a representative experiment was shown.

2.6. Statistical analysis

Statistical significance between two groups was tested using the unpaired Student's *t*-test. Differences were considered statistically significant at a value of $P < 0.05$.

3. Results

3.1. Halothane blocks hypoxia-induced HIF-1-dependent gene expression

To examine the effect of halothane on hypoxia-induced HIF-1 activity, Hep3B cells were subjected to reporter assay using p2.1 containing an HRE from the *ENO1* gene. Cells were exposed to 20% or 1% O_2 for 16 h with or without 1% halothane and then subjected to luciferase assays. Halothane suppressed hypoxia-induced reporter gene transcription in a dose-dependent manner (Fig. 1A, upper panel). Halothane also suppressed hypoxia-induced gene expression dependent on a *VEGF*-derived HRE (Fig. 1A, lower panel). In both reporter systems, the suppressive effect of halothane was maximal at a concentration of 1%. Halothane did not significantly affect gene expression in cells exposed to 20% O_2. It also did not affect gene expression of p2.4 or P11m, which contain mutations that prevent HIF-1 binding. Halothane did not significantly affect the activity of β-galactosidase (Fig. 1B). These results demonstrate that halothane has no effect on basal transcription but specifically inhibits HRE-driven gene transcription.

3.2. Halothane blocks hypoxia-induced HIF-1α protein expression

The biological activity of HIF-1 is determined by the protein expression and transcriptional activity of the HIF-1α subunit in Hep3B cells [3]. We exposed Hep3B cells to hypoxia to examine whether halothane affects HIF-1α protein expression. 0.5% halothane partially suppressed the induction of HIF-1α

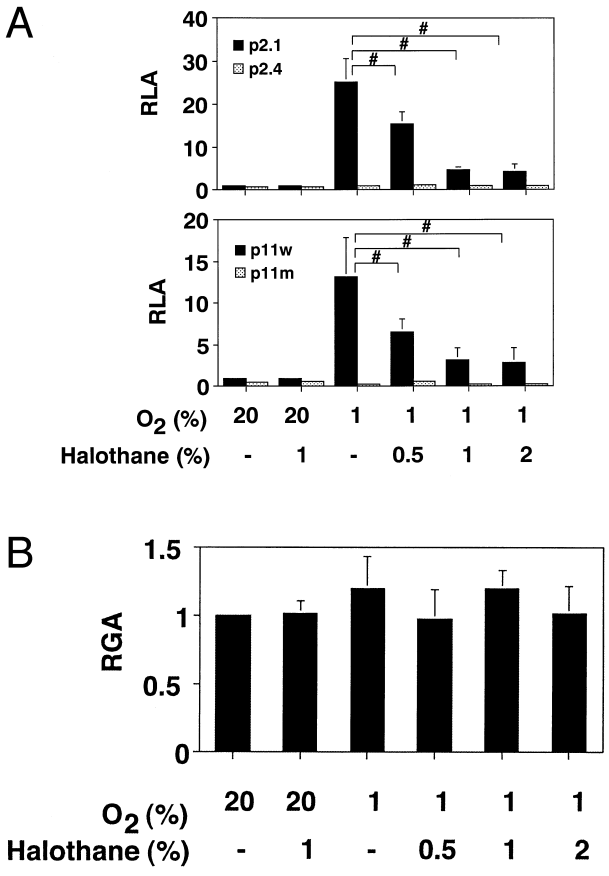

Fig. 1. Effect of halothane on hypoxia-induced HIF-1-mediated gene transcription. Hep3B cells were transfected with pSV40-β-galactosidase (100 ng), p2.1 or p2.4 (upper panel) (200 ng) or P11w or P11m (200 ng) (lower panel). Cells were exposed to 20% or 1% O_2 for 16 h with or without halothane. The relative fold induction of luciferase activity was calculated after normalization dividing the luciferase activity by β-galactosidase activity (A). 10 μl of lysate was subjected to β-galactosidase assay (B). The results are the means ± S.D. and are presented as fold increases in luciferase activity over the baseline seen at 20% O_2 without halothane. $^{\#}P < 0.05$. RLA, relative luciferase activity; RGA, relative galactosidase activity.

expression in response to hypoxia (Fig. 2A, lane 1). 1% or 2% halothane almost completely suppressed the hypoxia-induced accumulation of HIF-1α protein (lanes 2 and 3, respectively). As in the case of HRE-dependent transcription, 1% halothane is sufficient to block the accumulation of HIF-1α protein in Hep3B cells.

We examined whether this suppressive effect of halothane on HIF-1 activity was reversible. Hep3B cells were treated with 1% halothane for 8 h, recovered in air in the absence of halothane for 4 h and then were exposed to 1% O_2 for 4 h. No significant difference in HIF-1α protein accumulation was observed in halothane-treated and halothane-non-treated cells (Fig. 2B).

3.3. Halothane blocks hypoxia-induced transactivation by HIF-1α

There are two independent transactivation domains (TADs) present in HIF-1α. Because it has been shown that steady-state levels of fusion proteins consisting of the GAL4 DNA binding domain fused to HIF-1α residues 531–826 are similar under hypoxic and non-hypoxic conditions (in contrast to

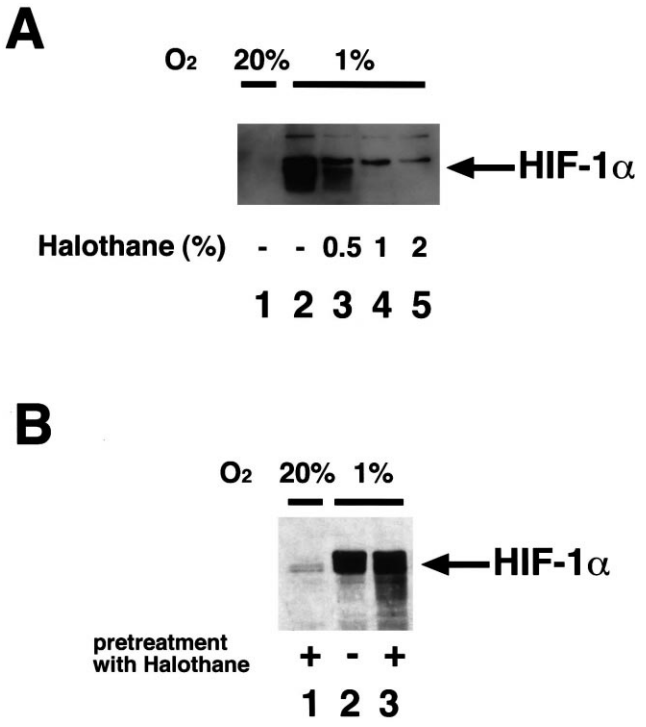

Fig. 2. Effects of halothane on hypoxia-induced HIF-1α protein expression. A: Hep3B cells were exposed to 20% or 1% O_2 with or without halothane for 4 h before nuclear extract preparation. 30-μg aliquots were subjected to Western blotting. B: Hep3B cells were exposed to 1% halothane for 8 h and recovered in air for 4 h (lanes 1 and 3). The treated cells were exposed to 1% (lane 3) or 20% O_2 (lane 1) for 4 h, harvested, and 30 μg of nuclear extracts was subjected to Western blotting.

endogenous HIF-1α protein expression which is restrictedly regulated by O_2 tension) [9], this GAL4-HIF-1α fusion construct can be used to examine the transcriptional activity of HIF-1α independently of its protein expression level. 1% halothane inhibited hypoxia-induced transactivation mediated by GAL4-HIF-1α(531–826) (Fig. 3). pCMV-GAL4-VP16-dependent gene expression was not affected by 1% halothane. This suggests that neither GAL4 DNA binding activity nor the basal transcriptional machinery was affected by halothane. Taken together with data in Fig. 1, the data also indicate that luciferase activity is not affected by halothane in our experimental system. Thus, halothane specifically inhibits hypoxia-induced HIF-1α TAD function.

3.4. Halothane blocks $CoCl_2$- or DFX-induced HIF-1 activation

As in the case of hypoxia, $CoCl_2$ and an iron chelator, DFX, have been established to activate HIF-1 by blocking the interaction of HIF-1α with von Hippel–Lindau tumor suppressor protein (VHL) [16–19]. We examined whether halothane affected $CoCl_2$- or DFX-induced HIF-1 activation. As shown in Fig. 4A, halothane suppressed $CoCl_2$- or DFX-induced HIF-1α protein accumulation. In addition, halothane suppressed $CoCl_2$- or DFX-induced HRE-dependent gene expression (Fig. 4B).

4. Discussion

In this report, we have shown that halothane is a reversible suppressor of HIF-1 activity within a range of clinically relevant doses. Our data have clearly demonstrated that halothane blocked luciferase gene expression driven by an HRE from either the *ENO1* or *VEGF* gene and that it blocked both HIF-1α protein accumulation and transactivation mediated by HIF-1α. Halothane did not significantly affect luciferase or β-galactosidase enzymatic activity directly. Although there are reports that volatile anesthetics including halothane inhibit firefly luciferase activity directly in vitro [20], data in Figs. 1 and 3 demonstrated that our experimental system was not affected by direct effects on luciferase protein.

HIF-1 is expressed in the cells and tissues of mammals. The HIF-1β subunit is also a partner for the arylhydrocarbon receptor and the bHLH-PAS proteins and is abundantly expressed independently of oxygen tension. In contrast, HIF-1α cannot be detected unless cells are challenged by hypoxia. Above a critical intracellular oxygen tension, HIF-1α is rapidly degraded in proteasomes after its ubiquitination [3]. HIF-1α contains an oxygen-dependent degradation domain within which is a highly conserved region containing a binding site for VHL [3]. VHL organizes the assembly of a complex that activates a ubiquitin E3 ligase, which then ubiquitinates HIF-1α, targeting it for degradation. Besides hypoxia, HIF-1 can also be induced by transition metal cations such as cobalt and by reagents that chelate iron [3]. Three recent reports proposed that a proline residue of HIF-1α is hydroxylated by a putative proline hydroxylase which requires oxygen and iron for its activation and that this modification plays a critical role in ubiquitination of HIF-1α under normoxic conditions [17–19]. Remarkably, halothane suppressed hypoxia-, $CoCl_2$- or DFX-induced HIF-1α protein accumulation. Halothane influences enzymatic activities of heme proteins including nitric oxide synthase and certain subtypes of NADPH oxidase [21,22].

Two gaseous molecules, nitric oxide and carbon monoxide, suppress hypoxia-induced HIF-1α protein accumulation and HIF-1α transactivation [23–25]. HIF-1α activation by treatment with $CoCl_2$ or DFX is also blocked by NO donors.

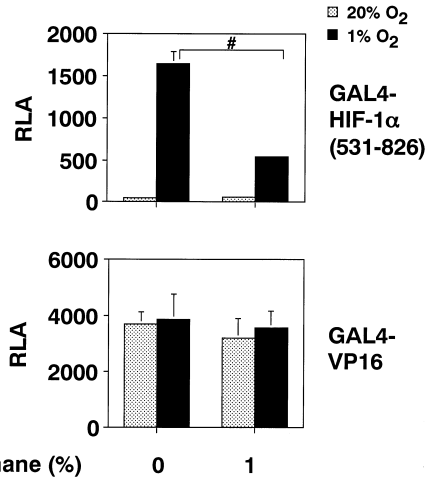

Fig. 3. Effects of halothane on hypoxia-induced HIF-1α TAD function. Construct pGAL4-HIF-1α(531–826) was analyzed for its ability to transactivate reporter gene ptk-GALpx3-LUC. Hep3B cells were co-transfected with pGAL4-HIF-1α(531–826) (100 ng), ptk-GALpx3-LUC (100 ng), and pSV40-β-galactosidase (100 ng). Cells were exposed to 20% O_2, or 1% O_2 for 16 h and then harvested. The results are the means ± S.D. and are presented as fold increases in luciferase activity over the baseline seen at 20% O_2 without treatment with halothane. $^{\#}P < 0.05$. RLA, relative luciferase activity.

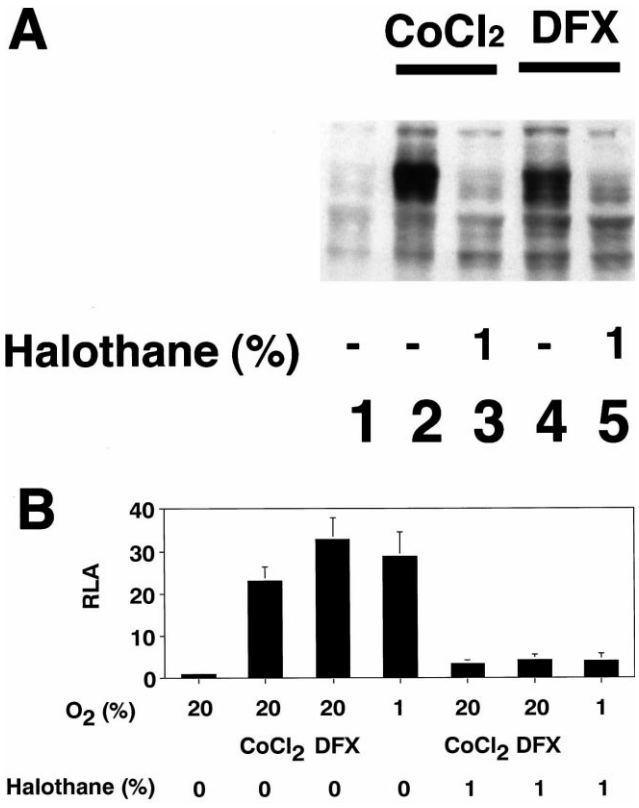

Fig. 4. Effect of halothane on $CoCl_2$- or DFX-induced HIF-1-mediated gene transcription. A: Hep3B cells were exposed to 100 μM $CoCl_2$ or 100 μM DFX with or without halothane for 4 h before nuclear extract preparation. 30-μg aliquots were fractionated by SDS–PAGE and subjected to immunoblot assay using an anti-HIF-1α antibody. B: Hep3B cells were transfected with pSV40-β-galactosidase, p2.1. Transfected cells were exposed to 100 μM $CoCl_2$ or 100 μM DFX for 16 h with or without halothane. The results are the means ± S.D. and are presented as fold increases in luciferase activity over the baseline seen at 20% O_2 without treatment with halothane. RLA, relative luciferase activity.

Interestingly, certain types of NO donors such as NOC-18 and GSNO can also activate HIF-1 under non-hypoxic conditions [26–28]. As shown in this report, halothane does not induce HIF-1 activation under non-hypoxic conditions. Therefore, halothane may block HIF-1 activation by different molecular mechanisms from those involving NO and CO.

In in situ rat liver HIF-1α protein is expressed predominantly in the perivenous zone, in which O_2 concentrations range from 30 to 35 mm Hg (4–5%) [29,30]. Taking account of the evidence that HIF-1 activation occurs at less than 5% O_2 [31], the expression pattern may be correlated with the O_2 concentration gradient in the liver sinusoids. The switch from aerobic to anaerobic metabolism during chronic hypoxia is regulated by HIF-1 and maintenance of cellular lactic acid concentration and cellular proliferation under hypoxic conditions requires HIF-1 [32]. In the liver, cell proliferation during regeneration after partial hepatectomy starts from the periportal areas [33]. Taken together with its essential role in the metabolic switch, HIF-1 may play a critical role in regeneration after partial liver resection. Because HIF-1 activation is observed within 2 min after onset of hypoxia [34], anesthesia with halothane several hours during operation may affect metabolic function and liver regeneration.

In summary, we have shown that halothane reversibly blocks cellular hypoxic responses mediated by HIF-1. Because the involvement of HIF-1 in hypoxic response is not limited to the liver, investigations of the effects of halothane in other tissues or organs, such as brain, kidney, lung and blood vessels, and of other anesthetics both volatile and those administered intravenously are indicated. Those studies may provide new insights into the mechanisms by which HIF-1 activity is regulated by cellular O_2 concentrations. In addition, they may indicate the involvement of HIF-1 inhibition in the pathophysiology of halothane-induced hepatotoxicity.

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