

Up-regulation of Hypoxia Inducible Factor 1α by Isoflurane in Hep3B Cells

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Background: **The volatile anesthetic isoflurane induces hypoxia inducible factor (HIF)-1–responsive genes heme oxygenase 1, inducible nitric oxide synthase, and vascular endothelial growth factor (VEGF) expression. Little is known about the extent to which induction of HIF-1α is affected by isoflurane.**

Methods: **Hep3B cells were exposed to isoflurane at various concentrations (0.5–4%) or for different time periods (2–8 h) at 37°C. HIF-1α gene expression and transcriptional activity, heme oxygenase 1, inducible nitric oxide synthase, and VEGF gene expression were quantified.**

Results: **Isoflurane induced a time- and concentration-dependent increase in HIF-1α protein but not for HIF-1α messenger RNA (mRNA) in Hep3B cells. The maximal increase was induced by 2% isoflurane, and the cells incubated with 2% isoflurane for 4–8 h expressed the highest protein. Similarly, HIF-1α transcriptional activity was higher in Hep3B cells exposed to 2% isoflurane for 16 h than that in control cells. The combination of 2% isoflurane and desferrioxamine, a hypoxia mimetic, caused a higher level of HIF-1α protein than that induced by 2% isoflurane alone. Reoxygenation and inhibitor of proteasome pathway MG132 did not affect the isoflurane-induced HIF-1α protein accumulation. Cycloheximide, an inhibitor for protein synthesis, completely abrogated the induction of HIF-1α protein by isoflurane. Isoflurane stimulated heme oxygenase 1, inducible nitric oxide synthase, and VEGF mRNA expression in a concentration-dependent manner, and inactivation of HIF-1α attenuated the induction of VEGF mRNA by isoflurane.**

Conclusion: **Isoflurane can up-regulate HIF-1α and enhance HIF-1–responsive genes heme oxygenase 1, inducible nitric oxide synthase, and VEGF mRNA expression in Hep3B cells. The induction of HIF-1α by isoflurane does not involve protein degradation but depends on translation pathway.**

HYPOXIA induces a series of adaptive physiologic responses. The response of cells is characterized by specific alterations in the expression of a large number of genes. Hypoxia inducible factor (HIF)-1, a transcription factor of the basic helix-loop-helix-Per-Arnt-Sim superfamily, is of central importance for the control of the expression of most of these genes.[1] Among the critical physiologic process regulated by HIF-1 target genes are erythropoiesis, angiogenesis, and glycolysis, which are examples of systemic, local tissue, and intracellular adaptive responses to hypoxia, respectively. This heterodimeric transcriptional factor consists of two subunits, HIF-1α and HIF-1β. HIF-1α is the specific and oxygen-regulated subunit of the HIF-1 complex and determines the level of HIF-1 activity, whereas HIF-1β, also termed the aryl hydrocarbon nuclear receptor translocator, is constitutively expressed.[2] Under hypoxic conditions, HIF-1α accumulates, translocates into the nucleus, and forms an active HIF-1 complex that takes part in the transcriptional activation of hypoxia-responsive genes through binding to the hypoxia-response element (HRE) in the promoter or enhancer region of these genes.[3] The regulation of HIF-1α activity occurs at multiple levels *in vivo*.[1] Among those, the mechanisms regulating HIF-1α protein expression and transcriptional activity have been most extensively analyzed. An important recent advance has been that human HIF-1α is constitutively hydroxylated at Pro-402 and Pro-564 by prolyl hydroxylases in normoxia, resulting in recognition by the von Hippel-Lindau ubiquitin ligase that leads to polyubiquitination and proteasomal degradation.[4,5]

It seems clear that HIF-1α regulation is complex and that factors other than oxygen might contribute to the activity of this transcription factor. Isoflurane-induced protection against ischemic–hypoxic injury has been widely accepted, but the precise mechanisms responsible for this effect remain unclear despite extensive study. It is clear that isoflurane can induce HIF-1–responsive genes such as inducible nitric oxide synthase (iNOS),[6–9] heme oxygenase (HO)-1 in liver,[10,11] and vascular endothelial growth factor (VEGF) in lung.[12] The mechanism of this inducing action is not fully elucidated. In this work, we decided to further study the regulation of HIF-1α by volatile anesthetic isoflurane in Hep3B cells, a widely used model for studies of hypoxia-related gene expression. Here we show that the level of HIF-1α in Hep3B cells is enhanced by the presence of isoflurane. A mechanism that might explain this effect is presented, and the implications are discussed.

Materials and Methods

No human or animal subjects were used for this study. All reagents were obtained from Sigma Chemical (St. Louis, MO) unless specified in the text. Hep3B cells (American Type Culture Collection, Manassas, VA) were maintained in minimum medium at 37°C with Earle's salts supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), essential amino acids, pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were used for experiments when they were 80% confluent. Hypoxia and normoxia protocol was set up as we described previously.[13] Briefly, on the day of the experiment, the medium was replaced with a thin layer of fresh

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Table 1. Nucleotide Sequences of PCR Primers Used in Real-time Quantitative RT-PCR

Primer	Orientation	Sequence	PCR Product Size, Base Pairs	Gene Bank Accession No.
HIF-1α	Sense	5′-CCAGCAGACTCAAATACAAGAACC-3′	138	NM_001530
	Antisense	5′-TGTATGTGGGTAGGAGATGGAGAT-3′		
VEGF	Sense	5′-CCTTGCCTTGCTGCTCTAC-3′	80	NM_003376
	Antisense	5′-GATGATTCTGCCCTCCTCC-3′		
HO-1	Sense	5′-CTATGTGAAGCGGCTCCACG-3′	348	NM_002133
	Antisense	5′-GCTCTGGTCCTTGGTGTCAT-3′		
iNOS	Sense	5′-GGTGGAAGCAGTAACAAAGGA-3′	231	NM_000625
	Antisense	5′-GACCTGATGTTGCCGTTGTTG-3′		
β-Actin	Sense	5′-GCTCGTCGTCGACAACGGCTC-3′	353	NM_001101
	Antisense	5′-CAAACATGATCTGGGTCATCTTCTC-3′		

HIF-1α = hypoxia inducible factor 1α; HO-1 = heme oxygenase 1; iNOS = inducible nitric oxide synthase; PCR = polymerase chain reaction; RT-PCR = reverse-transcription polymerase chain reaction; VEGF = vascular endothelial growth factor.

medium (0.15 ml/cm^2) to decrease the diffusion distance of the ambient gas. Hypoxia was produced by placing cells in a humidified airtight chamber (approximately 5 l in volume) with inflow and outflow valves, and hypoxic gas mixture (1% O_2, 5% CO_2, and 94% N_2) was delivered at 10 l/min for 25 min. The airtight incubator was kept at 37°C for preset time periods, whereas that which received no intervention was kept under normoxia condition (21% O_2, 5% CO_2, and 74% N_2) for the preset time periods.

Isoflurane Exposure

Isoflurane exposure was performed as described previously.[14,15] Briefly, cell dishes were kept in the airtight chamber housed within a water jacketed incubator maintained at 37°C. An in-line calibrated anesthetic agent vaporizer was used to deliver isoflurane to the gas phase of the culture wells. Normoxic gas was administered at a flow rate of 3 l/min, until the appropriate effluent concentration of the anesthetic was achieved. This required approximately 10–20 min. The flow rate was then decreased and maintained at 900 ml/min for preset periods at 37°C. Effluent isoflurane, oxygen, and carbon dioxide concentrations were continuously monitored *via* a sampling port connected to an anesthetic agent analyzer (5250, RGM; Datex-Ohmeda, Madison, WI). The pH of the medium before and after exposure was 7.4. The agents applied during the incubation, in addition to various concentrations of isoflurane, included cobalt chloride and desferrioxamine (hypoxia mimetic, 100 μM), cycloheximide (inhibitor of protein translation, 100 μM), and MG132 (a proteasome inhibitor, causes cells to accumulate HIF-1α protein, 50 μM). In experiments involving these compounds, they were added to the culture media just before the start of hypoxia or isoflurane stimulation, and the media was replaced at the end of the stimulation.

Protein Extraction and Western Analysis. Whole cell extracts were prepared as we have described previously.[13] A total of 100 μg protein was loaded onto a 10% SDS–polyacrylamide gel, and after electrophoresis, it was blotted onto nitrocellulose membranes. The primary rabbit anti-human HIF-1α (Upstate, Charlottesville, VA) polyclonal antibodies were used at 1:1,000 dilution, and anti-HIF-1β monoclonal antibody (Novus Biologicals, Littleton, CO) was used at 1:1,000 dilution. The anti-rabbit immunoglobulin G secondary antibody (KPL, Gaithersburg, MD) was used at 1:2,000 dilution, and the signal was analyzed by enhanced chemiluminescence (Chemicon, Temecula, CA). The intensity of each band was quantified with the software Quantity One-4.2.3 (Bio-Rad, Hercules, CA) and normalized to HIF-1β by density analysis.

Reverse Transcription and Real-time Quantitative Polymerase Chain Reaction

Cells were harvested and RNA was isolated as we described previously.[13] Real-time quantitative polymerase chain reaction (PCR) estimation of messenger RNA (mRNA) levels was performed as described previously.[16] Real-time quantitative PCR was performed by using SYBR green Ÿ as fluorescent dye (TaKaRa Biotechnology, Dalian, China). Primers were commercially obtained (Shanghai Sangon Biologic Engineering & Technology and Service Co. Ltd., Shanghai, China) (table 1). Amplification and detection were performed by using an ABI PRISM 7700 detection system (Applied Biosystems, Foster City, CA) as follows: 1 cycle at 95°C for 10 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s. Real-time PCR was performed in triplicate reactions with 20 ng complementary DNA in a final volume of 10 μl containing 1 $\times$ SYBR Green Master Mix and 200 nM of both primers. Agarose gel electrophoresis, purification, and DNA sequencing confirmed the identity of the PCR products. Reference gene β-actin, which was not altered by isoflurane or any other treatment applied in this study (data not shown), was used for normalization of the expression data. All reverse-transcription and real-time quantitative PCR was performed in triplicate from three separate culture dishes.

Enzyme-linked Immunosorbent Assay

The levels of VEGF protein secreted by the cells in the medium were determined by the VEGF enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). In brief, subconfluent cells were cultured in serum-free medium under normoxia overnight, followed by incubation with indicated stimuli for 16 h. The medium was collected, and VEGF protein concentrations were measured according to the manufacturer's instructions. The results were normalized to the number of cells per plate.

HIF-1α Transcriptional Activity Assay

Transient transfection of a luciferase reporter driven by HRE is one of the most sensitive systems for the measurement of HIF-1 activity. The Dual-luciferase Reporter Assay System exploits the differing biochemical requirements for luminescence of the firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) luciferase proteins. This allows for the sequential quantitative measurement of both luciferase activities in a single protein extract. The luciferase reporter plasmids (pGL3-luc) harboring HRE from human VEGF gene promoter region (−88/+54) and its HRE mutant were kindly provided by Amit Maity, M.D., Ph.D. (Assistant Professor of Radiation Oncology, Department of Radiation Oncology, University of Pennsylvania School of Medicine, Philadelphia, PA). The *Renilla* luciferase expression plasmid pRL-SV40 was obtained from Promega (Madison, WI).

Hep3B cells were plated 5×10^4cells/well on the day before transfection. Each plate was transfected with a mixture of 200 ng reporter gene plasmid and 50 ng control plasmid pRL-SV40 for transfection efficiency, premixed with Lipofectamine 2000 and Opti-MEMI reduced serum medium (Invitrogen, Shanghai, China). After 6 h, cells were recovered overnight and subsequently incubated with or without isoflurane under normoxic conditions with or without desferrioxamine for another 16 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).

RNA Interference Experiments

The small interference RNA (siRNA) oligonucleotide for HIF-1α was designed as described previously.[17] Sense and antisense RNA strands (5′-UGUGAGUUCGCAUCUUGA UdTdT-3′) and (5′-AUCAAGAUGCGAACUCACAdTdT-3′) with two 5′ deoxy-thymidine overhangs were commercially synthesized (KanChen Biology Co. Ltd., Shanghai, China) and annealed at a final concentration of 20 μM each by heating at 95°C for 1 min and incubating at 37°C for 1 h in annealing buffer (20 mM potassium acetate, 6 mM HEPES-KOH, pH 7.4, and 0.4 mM magnesium acetate). Transfection of siRNA was performed at a concentration of 100 nM using Lipofectamine 2000. As a control for HIF-1α–siRNA, we used either a corresponding random siRNA sequence(scrambled-siRNA:sense5′-AGUUCAACGACCA-GUAGU CdTdT-3′ and antisense 5′-GACUACUGGUCG-UUGAdTdT-3′) or in case of luciferase reporter gene assay also luciferase siRNA (sense 5′-CUUACGCUGAGUACUUC-GAdTdT-3′ and antisense 5′-UCGAAGUACUCAGCGUAAG-dTdT-3′) targeting firefly luciferase.[18] Control cells were transfected without oligonucleotides under the same conditions. After transfection, the cells were split in aliquots and grown in six-well dishes for 24 h and then exposed to normoxic or indicated condition for preset time periods. Cells were lysed and total RNA or protein was extracted as described above.

Statistical Analysis

One-way analysis of variance was used to compare mean values across multiple treatment groups with a Tukey *post hoc* multiple comparison test. In all cases, statistical significance was defined as $P < 0.05$. All data are expressed throughout the text as mean ± SD.

Results

Isoflurane Stimulates HIF-1α Protein Accumulation in Hep3B Cells

The biologic activity of HIF-1 is determined by the protein expression and transactivation activity of HIF-1α subunit. Isoflurane stimulated HIF-1α accumulation in a concentration-dependent manner in Hep3B cells; an increase could be seen at 1%, whereas maximal induction was attained at 2% (fig. 1A). In contrast, HIF-1β expression remained unchanged by isoflurane or any other treatment applied in the study, attesting to the fact that HIF-1β is constitutively expressed. So we used it as a loading control as described previously.[19] Time course studies were then performed on Hep3B cells stimulated with 2% isoflurane. An increase could be seen after a 2-h stimulation, and maximal induction was attained between 4 and 8 h in the presence of isoflurane (fig. 1B). However, this induction is slow as compared with the hypoxic induction of HIF-1α in these same cells. HIF-1α induction in Hep3B cells had already attained a maximum after a 2-h stimulation under hypoxia (fig. 1B). This indicates that different mechanisms might be implicated in the activation of HIF-1α by hypoxia and by isoflurane in these cells. HIF-1α is predominantly regulated at the protein level by oxygen-dependent prolyl hydroxylases. Cobalt chloride and desferrioxamine cause accumulation of HIF-1α protein through inhibition of prolyl hydroxylases and thus mimic hypoxic signaling.[20] As a positive control, we stimulated Hep3B cells with these reagents and isoflurane for the same period of time. HIF-1α protein level in normoxic cells was increased to a similar level by desferrioxamine (100 μM), cobalt chloride (100 μM), and 2% isoflurane and hypoxia (1% O_2) for 6 h (fig. 1C). The combination of isoflurane and desferrioxamine

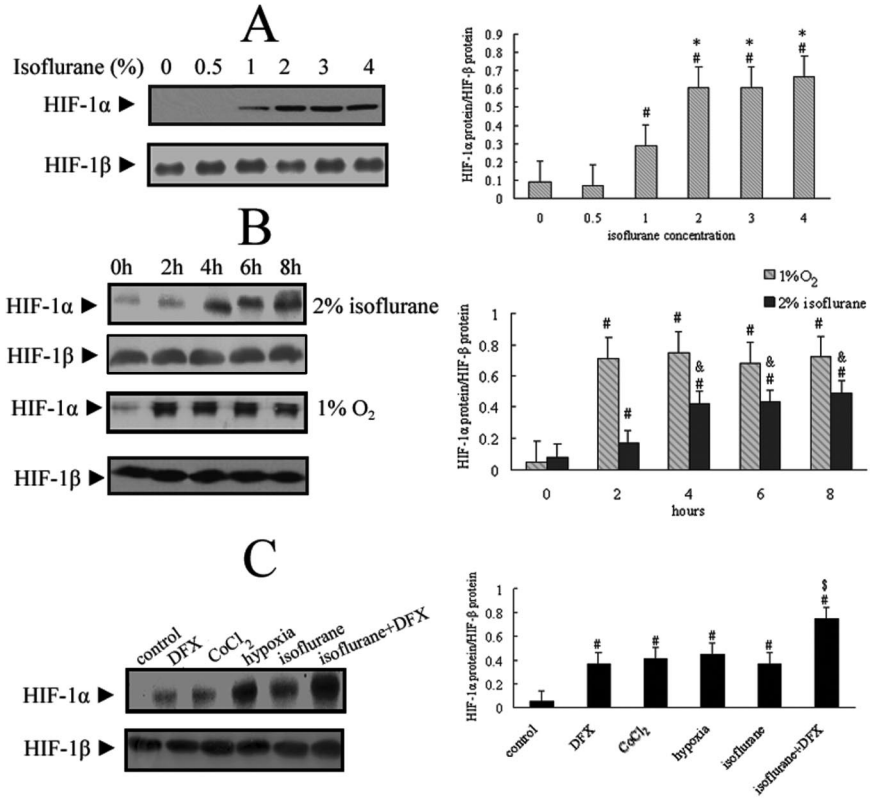

Fig. 1. Isoflurane induces hypoxia inducible factor (HIF)-1α protein accumulation. Hep3B cells were incubated under normoxic (21% O_2) conditions in the presence of various concentrations of isoflurane for 6 h (*A*), stimulated with 2% isoflurane–1% O_2 for indicated periods of time (*B*), or stimulated with indicated stimuli for 6 h (*C*), and HIF-1α and HIF-1β protein levels were analyzed. *Right panel* shows densitometric quantification of Western blot. Data are presented as mean ± SD of three different experiments. # $P < 0.05$ *versus* control (normoxic untreated cells). * $P < 0.05$ *versus* 1% isoflurane. & $P < 0.05$ *versus* cells incubated with isoflurane for 2 h. $ $P < 0.05$ *versus* desferrioxamine (DFX), cobalt chloride ($CoCl_2$), hypoxia, isoflurane alone.

resulted in greater increases in HIF-1α protein levels than those seen with either treatment alone. Desferrioxamine-treated cells were used in place of hypoxic cells because reoxygenation occurred when isoflurane was added. Taken together, these results show that the incubation of Hep3B cells with isoflurane results in a time- and concentration-dependent elevation of HIF-1α protein levels.

Translation-dependent Pathway but Not the Inhibition of Degradation Is Required for the Induction of HIF-1α by Isoflurane

To obtain a better understanding of the process involved in HIF-1α accumulation in response to isoflurane, we investigated the effect of isoflurane on the amount of HIF-1α mRNA. Hep3B cells were incubated with hypoxia–desferrioxamine–isoflurane for 6 h (fig. 2A) or stimulated with isoflurane for indicated time periods (fig. 2B). The change in HIF-1α mRNA did not reach statistical significance under all these conditions, suggesting that isoflurane does not regulate HIF-1α mRNA transcription just as hypoxia in Hep3B cells.[21] To elucidate posttranscriptional mechanisms, we examined whether isoflurane would stabilize HIF-1α. Therefore, Hep3B cells were exposed to desferrioxamine–isoflurane for 6 h and then returned to normoxia. Although isoflurane increased HIF-1α protein levels when incubated with desferrioxamine, it did not affect HIF-1α degradation on reoxygenation (fig. 3A). On the transition from desferri-

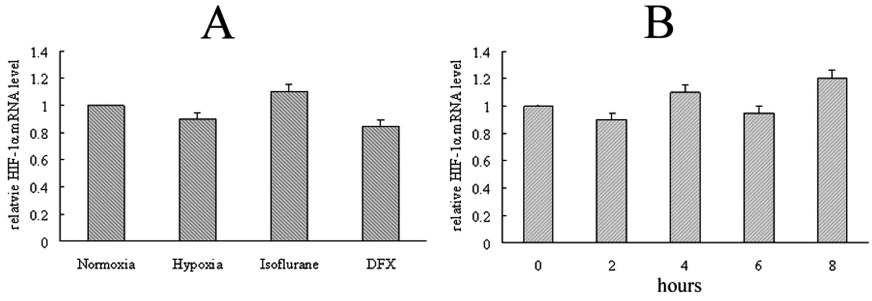

Fig. 2. Isoflurane does not affect hypoxia inducible factor (HIF)-1α messenger RNA (mRNA) levels. Hep3B cells were exposed to indicated stimuli for 6 h (*A*) or maintained under normoxic conditions in the presence of 2% isoflurane for indicated periods of time (*B*), and mRNA of HIF-1α and β-actin were analyzed. We assigned the value of 1 to the normalized HIF-1α mRNA level on control (normoxic untreated cells). The level of HIF-1α in the rest of samples is represented as fold over control. There is no statistically significant difference between controls and samples at any tested conditions. Data are presented as mean ± SD of three different experiments. DFX = desferrioxamine.

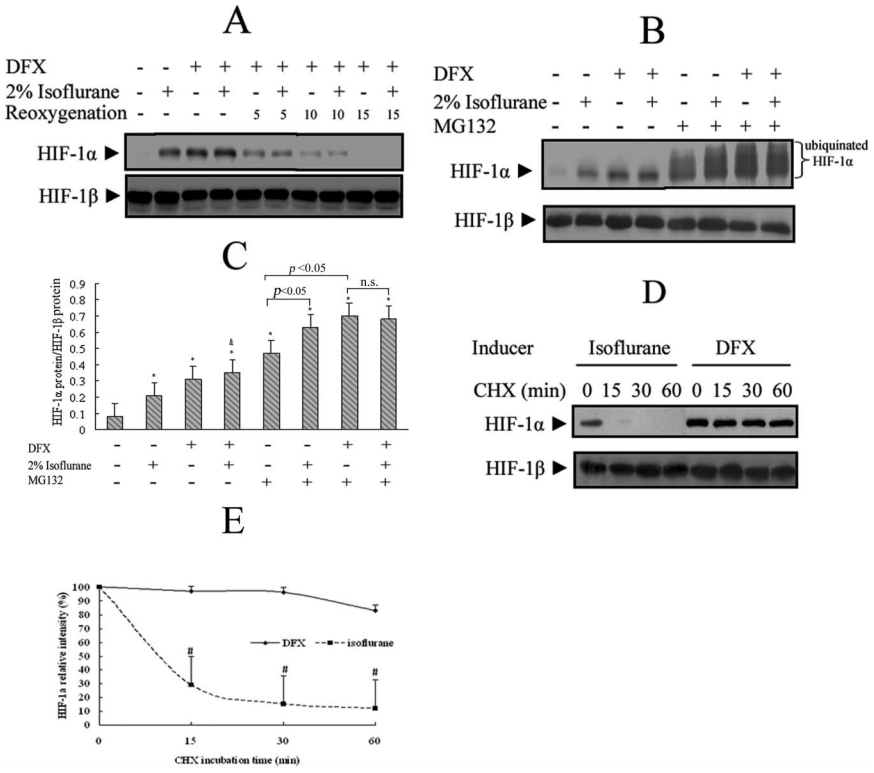

Fig. 3. Effects of isoflurane on hypoxia inducible factor (HIF)-1α does not involve protein degradation but is dependent on translation pathway. (*A*) Hep3B cells were exposed to indicated stimuli for 6 h, followed by replacing the DFX- and/or isoflurane-containing media with normoxic media and placing the cells in a normoxic incubator for indicated time periods (reoxygenation). (*B*) Hep3B cells were exposed to indicated stimuli for 6 h. (*C*) Levels of HIF-1α protein obtained in *B* were quantified. Data are presented as mean ± SD of three different experiments. * $P < 0.05$ *versus* normoxic cells without treatment. & $P < 0.05$ *versus* cells incubated with isoflurane or desferrioxamine (DFX) alone. n.s. = not significant. (*D*) Hep3B cells were exposed to indicated stimuli for 6 h, and then cycloheximide (CHX) was added to a final concentration of 100 μM for the indicated time periods. (*E*) Levels of HIF-1α protein obtained in *D* were quantified. The relative HIF-1α protein level at time zero was defined as 100%. The experiments were performed three times, and all data are presented as mean ± SD. # $P < 0.05$ *versus* the treatment of DFX.

oxamine to reoxygenation, HIF-1α completely disappeared within 15 min in desferrioxamine and desferrioxamine plus isoflurane–treated cells. Inhibition of the proteasome pathway by MG132 led to a high level of HIF-1α, and isoflurane induced HIF-1α accumulation in the presence of MG132. This elevated expression of HIF-1α in the presence of desferrioxamine and MG132 prevented us from detecting any effect of isoflurane on HIF-1α levels (figs. 3B and C). To analyze the isoflurane effect on HIF-1α synthesis, we performed a time course of HIF-1α disappearance in the presence of the protein translation inhibitor, cycloheximide. The addition of cycloheximide prevented the isoflurane-dependent induction of HIF-1α (fig. 3D). In the presence of cycloheximide, the half-life of HIF-1α was greater than 60 min in desferrioxamine-treated cells but less than 15 min in isoflurane-treated cells (fig. 3E). Together, these results suggest that isoflurane increases HIF-1α protein levels through a translation-dependent pathway but not *via* the inhibition of its degradation.

Isoflurane Stimulates HIF-1–responsive Gene Expression and Enhances VEGF through an HIF-1–dependent Pathway in Hep3B Cells

The data presented above show that the level of HIF-1α protein in Hep3B cells is increased upon isoflurane challenge, by promoting its translation. We next asked was the effect of isoflurane was on HIF-1 target genes. HO-1, iNOS, and VEGF are considered typically HIF-1–regulated genes.[22–24] Hypoxia significantly increased HO-1, iNOS, and VEGF mRNA expression, and isoflurane induced mRNA levels of these genes in a concentration-dependent manner (fig. 4A). Identical induction was observed between iNOS, HO-1, and VEGF by isoflurane. Thus, all further experiments were only performed on VEGF. Treatment of the cells with isoflurane for 16 h resulted in VEGF protein accumulation (fig. 4B).

We then tested the ability of isoflurane to activate an HIF-1–dependent luciferase reporter gene construct. Exposure of Hep3B cells to isoflurane or 1% O_2 for 16 h resulted in an increase of luciferase expression, as found for the endogenous VEGF gene (fig. 4C). The induction of luciferase expression by isoflurane disappeared when the reporter gene was under the control of a promoter lacking functional HRE element. Hence, the increment of HIF-1 target gene expression observed after isoflurane stimulation is attributable, at least in part, to the induction of HIF-1 acting through HRE. To further demonstrate that HIF-1α is required for the induction of VEGF mRNA expression in response to isoflurane, Hep3B cells were mock transfected or transfected with a $siRNA_{HIF\text{-}1\alpha}$ that targets HIF-1α mRNA for degradation. After transfection, the cells were exposed to desferrioxamine or isoflurane. As expected, Hep3B cells were efficiently cleared from HIF-1α protein, although a faint band was still visible in $siRNA_{HIF\text{-}1\alpha}$ and isoflurane-treated cells (fig. 5A). A subsequently performed luciferase reporter gene assay revealed a drastic loss of desferrioxamine and isoflurane-dependent induction (fig. 5B). VEGF mRNA levels were slightly reduced in cells treated with $siRNA_{HIF\text{-}1\alpha}$ in normoxia and dramatically reduced in isoflurane-induced VEGF mRNA expression (fig. 5C). However, the effect of $siRNA_{HIF\text{-}1\alpha}$ was modest (approximately 80%

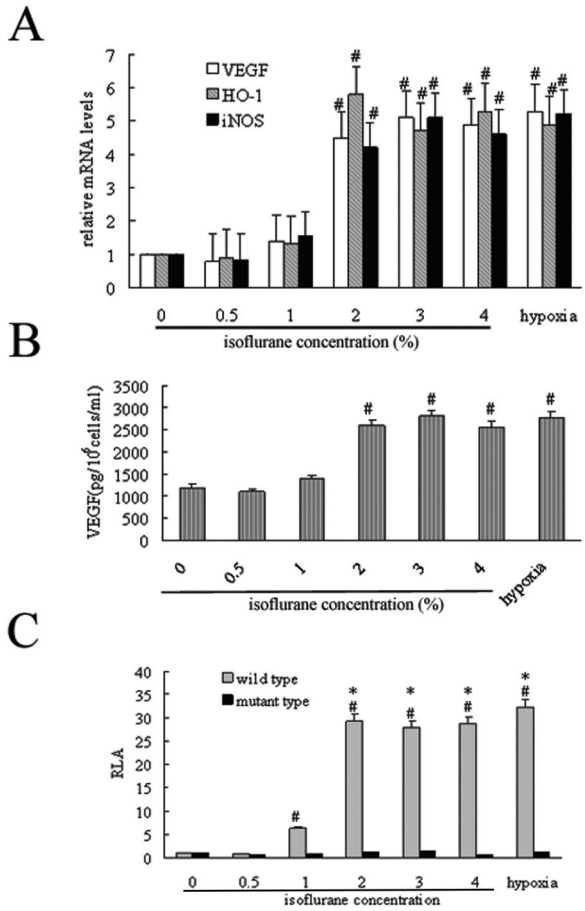

Fig. 4. Effects of isoflurane on hypoxia inducible factor (HIF)-1–responsive gene expression and HIF-1 transcriptional activity. (*A*) Hep3B cells were exposed to indicated stimuli for 6 h, and messenger RNA (mRNA) was analyzed. We assigned the value of 1 to the normalized HIF-1–responsive gene mRNA level on control (normoxic untreated cells). The levels of these genes in the rest of samples are represented as fold over control. (*B*) Hep3B cells were exposed to indicated stimuli for 16 h, and vascular endothelial growth factor (VEGF) protein was measured. (*C*) Hep3B cells were transiently transfected with reporter plasmids as described in the Materials and Methods. The ratio of firefly:*Renilla* luciferase activity was determined and normalized to the values obtained from normoxic, wild-type transfected cells to obtain the relative luciferase activity (RLA). # $P < 0.05$ *versus* control (normoxic untreated cells). * $P < 0.05$ *versus* 1% isoflurane. All data are presented as mean ± SD from three replicate experiments. HO-1 = heme oxygenase 1; iNOS = inducible nitric oxide synthase.

the control values), indicating that HIF-1 pathway is not the only mechanism involved in isoflurane-dependent induction of VEGF mRNA. As an additional control for siRNA transfection, we used luciferase-siRNA targeted against firefly luciferase. Luciferase-siRNA inhibited firefly-luciferase reporter gene expression more strongly when compared with the effects of HIF-1α-siRNA (fig. 5D).

Discussion

Isoflurane is known to affect the expression of HIF-1–responsive genes.[6–12] However, little is known about the extent to which induction of HIF-1, the master regulator of oxygen homeostasis, is affected by the presence of isoflurane. Herein, for the first time, we provide evidence that isoflurane up-regulates HIF-1α protein expression and activity in a time- and concentration-dependent manner; elevates HIF-1–responsive genes HO-1, iNOS, and VEGF mRNA levels; and induces VEGF through an HIF-1–dependent pathway in Hep3B cells. Moreover, in contrast to hypoxia, which is a major activator of HIF-1α, isoflurane does not regulate HIF-1α through the inhibition of its degradation but *via* a translation-dependent mechanism.

In this study, hypoxia treatment significantly increased HIF-1α protein levels in Hep3B cells, which is consistent with previous findings that hypoxia stimulates HIF-1α expression in the cell line.[21] We also found that isoflurane could increase HIF-1α protein level under normoxic conditions. The possibility that isoflurane might increase HIF-1α gene expression was rejected because HIF-1α mRNA levels were not elevated by isoflurane treatment. The lack of effect of isoflurane on transcriptional activation of HIF-1α was not surprising in view of the fact that hypoxic regulation of HIF-1α is also primarily determined by stabilization of HIF-1α protein.[2] Further, isoflurane did not affect HIF-1α degradation during reoxygenation. The findings indicated that isoflurane-inducible accumulation of HIF-1α protein might be due to an increase of HIF-1α protein synthesis. MG132 blocks the proteasomal degradation machinery, so the rate of HIF-1α accumulation is at large a function of the rate of HIF-1α synthesis in the presence of MG132. Isoflurane significantly enhanced HIF-1α protein levels in the presence of MG132 under normoxic conditions. The effect of isoflurane was abrogated by cycloheximide, an inhibitor of translation. A comparison of the half-life of HIF-1α in the presence of isoflurane and desferrioxamine shows that isoflurane does not stabilize the HIF-1α protein. Nevertheless, we cannot exclude the possibility that isoflurane can regulate the translation of a protein which inhibits HIF-1α degradation.

We also demonstrate that mRNA for HO-1, iNOS, and VEGF are elevated in the presence of isoflurane. Preinduction of HO-1 and application of carbon monoxide have been shown to prevent reperfusion injury after liver transplantation or after systemic ischemia in several experimental studies.[11,25] iNOS has been shown to play an important role in cardioprotection or neuroprotection induced by many stimuli including isoflurane.[7–9] It could be argued that the observed effect of isoflurane on HIF-1–responsive gene expression is independent of HIF-1 and through a response element other than HRE. To investigate this possibility, we transiently transfected Hep3B cells with a reporter plasmid construct expressing the firefly luciferase gene under the control of the wild-type HRE from VEGF gene promoter or an HRE-mutated version of this promoter. HIF-1α was indispens-

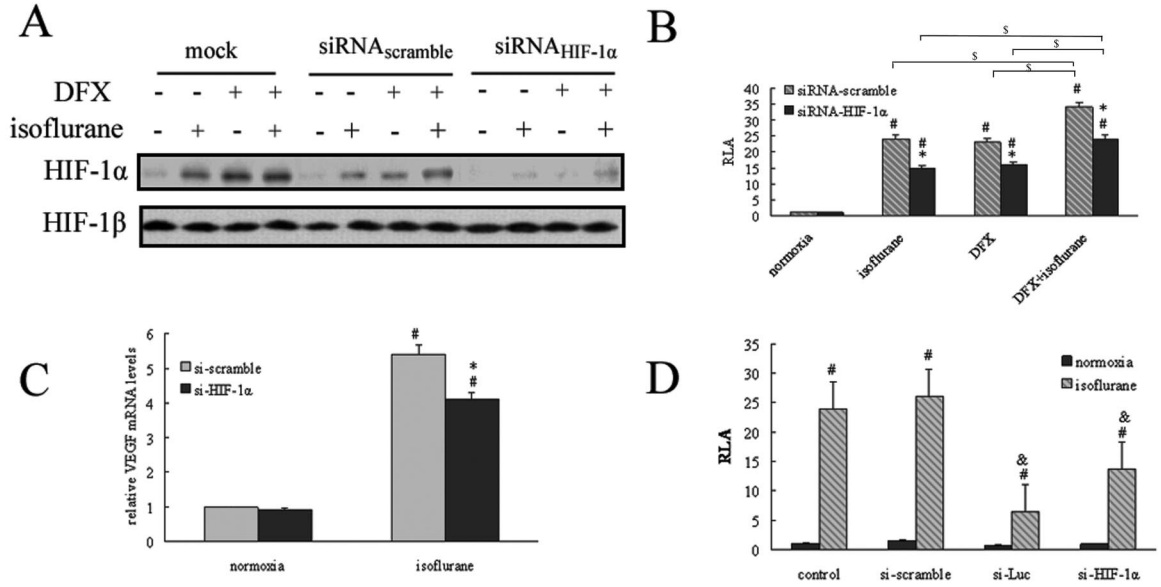

Fig. 5. Effects of hypoxia inducible factor (HIF)-1α RNA interference on HIF-1α protein level, HIF-1α transcriptional activity, and vascular endothelial growth factor (VEGF) messenger RNA (mRNA) expression. (*A*) Hep3B cells were mock transfected, or transfected with indicated small interference RNA (siRNA). A day after transfection, the cells were treated with indicated stimuli for 6 h, and HIF-1α and HIF-1β were determined by Western blot. (*B–D*) Hep3B cells were transiently transfected with hypoxia-response element (HRE)–luciferase reporter construct and/or indicated siRNAs as described in the Methods and Materials. Relative luciferase activity (RLA) and VEGF mRNA were analyzed. We assigned the value of 1 to the control (normoxia cells transfected with $siRNA_{scramble}$ or mock transfected); the values of the rest are represented as fold over control. # $P < 0.05$ *versus* corresponding control. * $P < 0.05$ *versus* si-scramble. & $P < 0.05$ *versus* mock transfection. $ $P < 0.05$. All data are presented as mean ± SD from three replicate experiments and are represented as fold over control. DFX = desferrioxamine; si-Luc = siRNA for luciferase.

able for isoflurane-induced stimulation of HIF-1–dependent reporter gene and target gene VEGF expression as shown by knocking down HIF-1α by siRNA. Activation of VEGF by HIF-1 contributes to cardioprotection.[26] Therefore, isoflurane alone increases HIF-1α protein levels under normoxic conditions and might potentiate hypoxia-induced HIF-1α expression. Increased synthesis by isoflurane cooperates with hypoxic stabilization and accumulation of HIF-1α that is due to inhibition of prolyl hydroxylase activity, and ensures enhanced expression of the HIF-1 target genes such as VEGF.

Recent studies have shown that a number of nonhypoxic stimuli could strongly increase HIF-1α in a cell-specific manner.[27] Interestingly, some of these increases have been shown to be equal to or greater than the hypoxic induction of HIF-1α. In this study, we show that in Hep3B cells, isoflurane is an excellent nonhypoxic stimulator and activator of the HIF-1 transcription factor. However, the signal transduction pathway of the induction by isoflurane remains unclear. Cumulative evidences suggest that isoflurane may exert protection against hypoxic–ischemic injury *via* the activation of phosphatidylinositol 3-kinase and intracellular calcium pathways. Isoflurane has been shown to activate phosphatidylinositol 3-kinase pathway,[28,29] and this signaling cascade has also been shown to play a major role in the induction of HIF-1α protein levels by different nonhypoxic stimuli.[30–36] Further, studies suggest that activation of phosphatidylinositol 3-kinase pathway preferentially increases HIF-1α protein translation.[35,36] Therefore, isoflurane may have induced HIF-1α *via* a phosphatidylinositol 3-kinase pathway. Other study demonstrated that calcium signaling stimulates translation of HIF-1α during hypoxia.[37] Isoflurane neuroprotection in hypoxic hippocampal slice cultures involves increases in intracellular calcium and mitogen-activated protein kinases.[38–40] Further investigation will be required to define whether either indicated signaling or other pathways are involved in this process.

It has been demonstrated that halothane reversibly blocks hypoxia-induced HIF-1α protein accumulation and transcriptional activity at clinically relevant doses.[41] Differential physiologic effects of volatile anesthetics are well known. For example, halothane and isoflurane produce strikingly different inotropic and vasodilatory effects.[42] In the experiment setting of healthy volunteers, isoflurane and halothane differentially affect the hypoxic chemoreflex loop.[43] Hepatic HO-1 expression was differentially regulated by several volatile anesthetics.[10] In addition, isoflurane induces protection of human myocardium against anoxia injury but not for halothane,[44,45] and isoflurane altered several genes involved with neurotransmitter transport, signaling and cellular structure, whereas halothane produced few detectable changes in primary cortical neurons.[46] Therefore, these previous studies as well our current study supports our hypothesis that the two anesthetics (isoflurane and halothane) genuinely differ in their actions on HIF-1 activation. These studies suggested, despite HIF being regulated

primarily by oxygen availability, other signals received by the cell modulate this response. Alternatively, these findings might indicate the regulation of HIF-1α by anesthetics is partly dependent on the stimulus used and the cell type studied.

In our study, isoflurane at concentration of 1–4% upregulated HIF-1α in Hep3B cells. These isoflurane concentrations are clinically relevant, because 1 minimum alveolar concentration (MAC) is 1.15%,[47] and isoflurane concentrations higher than 1 MAC are frequently used during anesthesia. In the liver, because of the unidirectional blood flow from the portal vein and hepatic artery to the central vein and because of the oxygen-consuming metabolic processes of the cells along the sinusoids, an oxygen gradient is formed. Therefore, the oxygen tension decreases from 65 mmHg in the periportal area to 35 mmHg in the perivenous area.[48] HIF-1α is present in mice liver under normoxic conditions and is further increased in response to systemic hypoxia (6% O_2).[49] Our data for HIF-1α were based on the human hepatoma cell line Hep3B cell, and its use to test the regulation of HIF-1α is of great interest. However, the immortalized cell line cannot be considered as equivalent for differentiated hepatocytes. It would be useful to test whether the regulatory events described in Hep3B cells might be applicable to normal human liver. Although it is not appropriate to extrapolate our results directly to *in vivo* conditions or clinical practice, our data suggest a potential site for volatile anesthetics to act in the liver. On the other hand, we treated cells with the anesthetics for rather short periods, whereas in the clinical setting, human bodies are usually exposed to anesthetics for longer periods than we used here. An increase in iNOS has been shown after the end of isoflurane anesthesia up to 24 h,[7] and changes in cerebral protein expression after desflurane anesthesia occur as rapidly as 3 h after the start of anesthesia and last up to 72 h.[50] Conversely, using organotypic hippocampal slices, it has been shown that isoflurane-induced neuronal degeneration is dependent on the duration of isoflurane exposure.[15,51] Further studies are needed from the point of view of time.

Our results suggest that HIF-1α should be involved in the activation of known hypoxic genes by isoflurane. However, we do not suggest that the HIF-1 pathway is the only mechanism involved in the activation of these genes. Multiple signaling pathways are activated by isoflurane in cells, and a number of pathways have been shown to play an important role in the activation of certain hypoxia-responsive genes. We believe that along with the other pathways and transcription factors, HIF-1 permits the maximal activation of these genes. Two recent studies by Hoetzel *et al.*,[10,11] based on the regulation of hepatoprotection enzyme HO-1 by isoflurane in liver, revealed exciting novel signal transduction pathway involving protein kinase C and phospholipase A_2. Unfortunately, HIF-1α levels were not determined in these studies. It is hoped that future work will elucidate the impact of HIF-1 on the protective effect of isoflurane in a variety of physiologic and pathophysiologic conditions.

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