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A Prolyl-hydroxylase Inhibitor, Ethyl-3,4-dihydroxybenzoate, Induces Haem Oxygenase-1 Expression in Human Cells Through a Mechanism Independent of Hypoxia-inducible Factor-1α

Bin Li, Kazuhisa Takeda*, Satoru Yokoyama† and Shigeki Shibahara

Department of Molecular Biology and Applied Physiology, Tohoku University School of Medicine, Sendai 980-8575, Japan

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Hypoxia-inducible factor (HIF)-1 is important for cellular homeostasis under hypoxia. Expression of haem oxygenase-1 (HO-1), an essential enzyme in haem catabolism, varies under hypoxia, depending on cell types. Here, we studied the role of HIF-1α, a component of HIF-1, in the regulation of HO-1 expression using three human cell lines: HeLa cervical cancer, and ARPE-19 and D407 retinal pigment epithelial cells. Under hypoxia (1% O_2), the expression of HO-1 mRNA was decreased in HeLa cells, increased in D407 cells, and unchanged in ARPE-19 cells, while HIF-1α protein was accumulated in these cell lines. Thus, HIF-1α is unlikely to function as a key regulator for HO-1 expression under hypoxia. We then used ethyl-3,4-dihydroxybenzoate (EDHB), an inhibitor of prolyl hydroxylases, to accumulate HIF-1α protein under normoxia. Treatment with EDHB (250–500 μM) increased HIF-1α protein levels in HeLa and D407 cells, but not in ARPE-19 cells, whereas EDHB at lower concentrations (50–100 μM) consistently induced HO-1 mRNA expression (about 20-fold) in these three cell lines. Moreover, EDHB increased the HO-1 gene promoter activity via the enhancer that lacks a HIF-1-binding site. In conclusion, the signals evoked by hypoxia and after EDHB treatment differentially regulate HO-1 mRNA expression through HIF-1α-independent mechanisms.

Key words: ethyl-3,4-dihydroxybenzoate, haem oxygenase-1, hypoxia, hypoxia-inducible factor-1α, prolyl-hydroxylase inhibitor.

Abbreviations: EDHB ethyl-3,4-dihydroxybenzoate; HIF-1α, hypoxia-inducible factor-1α; HO-1, haem oxygenase-1; HRE, hypoxia-responsive element; MARE, Maf recognition element; Nrf2, NF-E2-related factor-2; RPE, retinal pigment epithelium.

Hypoxaemia and the resulting tissue hypoxia are associated with various disorders, including severe asthma, pneumonia, chronic obstructive pulmonary disease and sleep apnoea syndrome, which account for common causes of death or disability in the developed world (*1*). Mammalian cells respond to hypoxia in part by increasing expression of many genes, including those that code for erythropoietin (*2*), vascular endothelial growth factor (*3*), adrenomedullin (*4*, *5*) and glycolytic enzymes (*6*, *7*), all of which cooperate to protect cells and tissues against the hypoxic insults. Hypoxia-inducible factor-1 (HIF-1) serves as a key regulator that induces the expression of most of these genes (*8*). HIF-1 consists of a heterodimer of two basic helix-loop-helix/Per-Arnt-Sim transcription factors: HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT) (*9*). Under normal oxygen conditions (normoxia), newly synthesized HIF-1α is rapidly degraded. Degradation is mediated by prolyl hydroxylases (*10*, *11*), a family of 2-oxoglutarate (2-OG)-dependent dioxygenases that hydroxylate conserved proline residues (Pro^{402} and Pro^{564}) in the oxygen-dependent degradation domain of HIF-1α (*12*). Recent biochemical studies have demonstrated that structural analogues of α-ketoglutarate can serve as potent competitive inhibitors of prolyl hydroxylase (*13*); namely, these analogues compete with α-ketoglutarate for the binding in the active site of the enzyme, thereby inhibiting the activity of prolyl hydroxylase (*14*). Ethyl-3,4-dihydroxybenzoate (EDHB) has been characterized as a specific cell-permeable inhibitor that competitively binds to both the ascorbate- and α-ketoglutarate-binding sites of prolyl hydroxylase (*15*, *16*).

The retinal pigment epithelium (RPE) normally functions under relatively high oxygen tensions, as RPE forms a single cell layer located between the retinal photoreceptors and the vascular-rich choroids. RPE is essential for visual function and survival of the photoreceptors. Conversely, impaired function of RPE may lead to loss of photoreceptors or retinal degeneration (*17*), which accounts for a major cause of ageing-dependent visual impairment and blindness in the developed world. Accordingly, RPE expresses various anti-oxidant enzymes, including haem oxygenase-1 (HO-1) and haem oxygenase-2 (HO-2) (*18*, *19*). Both HO-1 and HO-2 catalyse the same reaction in haem catabolism, cleaving haem

*To whom correspondence should be addressed. Tel: +81-22-717-8114; Fax: +81-22-717-8118, E-mail: ktakeda@mail.tains.tohoku.ac.jp
†Present address: Department of Pediatric Oncology, Dana630, Dana-Farber Cancer Institute, 44 Binney St., Boston MA 02115, USA.

to biliverdin IXα, carbon monoxide and iron (*20*). Biliverdin IXα is immediately reduced to bilirubin IXα during the last step of haem breakdown reaction (*21*). Expression of HO-1 mRNA is increased in human RPE cells by the substrate haem (*18*), transforming growth factor-β1 (*22*) or prostaglandin D_2 (*23*), and conversely decreased by interferon-γ (*24*).

Under hypoxia, expression of HO-1 is increased or decreased, depending on species and cell types (*1*, *25*). HO-1 expression is reduced under hypoxia in HeLa human cervical cancer cells (*26*), human umbilical vein endothelial cells (*27*), and A549 human lung cancer cells (*28*). On the other hand, hypoxia increased expression of HO-1 mRNA in D407 RPE cells but not in ARPE-19 RPE cells (*24*). Thus, hypoxia showed differential effects on HO-1 expression even in the same cell type. These results suggest that the mechanisms underlying HO-1 expression are complex in human cells and varied depending on a given microenvironment (*1*, *25*).

To explore the role of HIF-1α for the expression of HO-1 in human cells, we used EDHB, a prolyl-hydroxylase inhibitor, to accumulate HIF-1α under normoxia, and analysed the effects of EDHB on HO-1 expression in HeLa human cervical cancer cells and D407 and ARPE-19 human RPE cells. We have provided evidence that EDHB induces HO-1 expression in human cells through a mechanism that is independent of HIF-1α.

MATERIALS AND METHODS

Reagents—EDHB, Dulbecco's modified Eagle's medium (DMEM), MEM medium and RPMI 1640 medium were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) and other culture reagents were purchased from Gibco BRL (Grand Island, NY, USA). TRI Reagent, protease inhibitor cocktail and Nonidet P-40 were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-HIF-1α antibody was from Santa Cruz Biotechnology (Santa Cruz, USA), Anti-HO-2 antibody was from StressGen (Victoria, Canada), and anti-β-actin was from Sigma-Aldrich. Pfu Turbo DNA polymerase was purchased from Stratagene (La Jolla, CA, USA), DIG Northern Starter Kit and FuGENE 6 were purchased from Roche Diagnostics (Mannheim, Germany). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). All other chemicals were obtained from Sigma-Aldrich.

Cell Culture—The human RPE cell lines, ARPE-19 (*29*) and D407 (*30*), were provided by Leonard M. Hjelmeland (University of California, Davis, CA, USA) and Richard C. Hunt (University of South Carolina Medical School, Columbia, SC, USA), respectively. ARPE-19 cells were cultured in a 1:1 mixture of DMEM and Ham's F12, supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) (*29*). D407 RPE cells were cultured in DMEM high glucose containing 10% FBS and the antibiotics (*30*). HeLa cervical cancer cells were cultured in MEM medium and A549 human lung cancer cells were cultured in RPMI 1640 medium containing 10% FBS and antibiotics. To examine the effects of EDHB on the expression levels of HO-1 mRNA and protein, ARPE-19, D407, HeLa and A549 cells were grown to 70–80% confluence before they were incubated with vehicle (ethanol) or EDHB (10, 50, 100, 250 or 500 μM). For hypoxia experiments, ARPE-19 and D407 cells were cultured in a chamber equilibrated with 5% CO_2, 94% N_2 and 1% O_2. The cells were cultivated under normoxia or hypoxia for indicated hours, and harvested for extraction of RNA and protein.

Northern Blot Analysis—Total RNA was extracted from cultured ARPE-19, D407, HeLa and A549 cells using TRI Reagent. Total RNA (10 μg/lane) was fractionated by electrophoresis in a 1.0% agarose gel containing 2 M formaldehyde, transferred to a nylon membrane filter (Zeta-Probe membrane; Bio-Rad, Richmond, CA, USA), and fixed with a UV crosslinker (Stratalinker 1800; Stratagene). The northern probes used for haem oxygenase mRNAs were the XhoI/XbaI fragment of nucleotide positions 81–878 (GenBank accession number **X06985**) derived from the human HO-1 cDNA, pHHO1 (*31*), and the EcoRI/EcoRI fragment of nucleotide positions 85–939 (GenBank accession number **NM_002134)** derived from the human HO-2 cDNA, amplified fragment was cloned into pGEM-Teasy vector (Promega, Madison, WI, USA), and named pGEM-hHO-2. The expression of β-actin mRNA was examined as an internal control. The probe for β-actin mRNA was the SmaI/ScaI fragment (nucleotides 124–1,050) of a human β-actin cDNA provided by T. Yamamoto (Tohoku University, Sendai, Japan). These DNA fragments were labelled with [α-^{32}P]dCTP (Amersham Biosciences, Amersham, UK) by the random priming method and were used as hybridization probes. The RNA blot was hybridized with each ^{32}P-labelled probe, as described previously (*32*). Radioactive signals were detected by exposing the filters to X-ray films (X-AR5; Kodak, Rochester, NY, USA).

Northern blot analysis for HO-1 mRNA was also performed with the DIG Northern Starter Kit. For preparation of HO-1 RNA probe, the human HO-1 cDNA of positions 81–878 (GenBank accession number **X06985**) was amplified by PCR using Pfu Turbo DNA polymerase and then cloned into pCR-bluntII-TOPO (Invitrogen), named pCR-hHO1. SP6 RNA polymerase was used for transcription of RNA probe from pCR-hHO1, as detailed previously (*26*). Blots were scanned with a scanner interfaced to a computer and were analysed with the NIH ImageJ 1.38 program (http://rsb.info.nih.gov/nih-image/) to obtain intensity values for HO-1 mRNA and 18S levels in each sample. Relative HO-1 mRNA expression was obtained by dividing the intensity value for each sample with that of ethanol-treated sample at the same time point.

Western Blot Analysis—Cells were lysed in the buffer containing 20 mM Hepes (pH7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% protease inhibitor cocktail and 0.5% Nonidet P-40. Cell lysates (40 μg protein per lane) were fractionated by SDS–PAGE and blotted to a polyvinylidene fluoride membrane (Immobilon^{TM}-P, Millipore Corporation, Bedford, MA, USA) in the buffer containing 20% methanol, 48 mM Tris, 39 mM glycine and 0.037% SDS. The membranes (western blots) were treated overnight at 4°C in Tris-buffered saline (TBS) with 0.1% Tween-20

(TBS-T), containing 5% non-fat dried milk and were washed three times each for 10 min in TBS-T at room temperature. The proteins were probed with HO-1 antibody (*33*) at a dilution of 1 : 1,000 or β-actin antibody at a dilution of 1 : 5,000 for 1 h at room temperature. For detection of HO-2 protein, the blots were incubated overnight at 4°C with HO-2 antibody at a dilution of 1 : 2,000. HIF-1α antibody was used at a dilution of 1 : 500 for 1 h at room temperature. Immunoreactive proteins were detected with a western blot kit (Millipore Corporation). Blots were scanned with a scanner interfaced to a computer and were analyzed with the NIH ImageJ 1.38 program (http://rsb.info.nih.gov/nih-image/) to obtain intensity values for HIF-1α protein, HO-1 protein and β-actin levels in each sample. Relative HIF-1α or HO-1 protein expression was obtained by dividing the intensity value for each sample with that of ethanol-treated sample at the same time-point.

Luciferase Reporter Constructs—The human HO-1 gene-firefly luciferase constructs, phHOLUC45 contains the Maf recognition element (MARE) site GCTGAGTCA (positions −3,935 to −3,927), whereas phHOLUC40 lacks the MARE. phHOLUC-58 does not contain any promoter enhancer. The numbers of GT repeat are 30 in phHOLUC45 (*24*, *25*) and 23 in phHOLUC40 (*34*), respectively. The test plasmids, pRBGP2 and pRBGP4, contain three copies of the MARE and mutated MARE, respectively, in each promoter region linked to the luciferase gene (*35*). Reporter plasmids, pSV40 promoter-Epo HRE-Luc containing four copies of hypoxia-responsive element (HRE) and pSV40 promoter-Luc lacking HRE (*36*), were used as a positive and a negative control for hypoxic induction.

Transient Transfection and Luciferase Assays—ARPE-19 and HeLa cells were cultured in 12-well plates to 1.5×10^5 cells/well 1 day before transfection to reach 50–80% confluence. The cells were transfected with FuGENE 6 for 24 h using 20 ng of Renilla luciferase control plasmid, pRL-CMV vector (Promega), and 480 ng of each luciferase reporter construct per well. D407 cells were cultured in 24-well plates to 1.5×10^5 cells/well 1 day before transfection to reach 90–95% confluence. The cells were transfected with Lipofectamine 2000 by using 10 ng of control plasmid pRL-CMV, and 100 ng of each luciferase reporter construct per well. Following the 12 h incubation, cells were treated for 18 h with ethanol (vehicle) or EDHB (100 μM or 500 μM). A promoter-less construct (pGL3-basic) was used as a control. Expression of reporter gene and pRL-CMV was determined with the Dual-Luciferase™ Reporter Assay System (Promega).

Measurement of Haem Content—Haem contents in RPE cells (expressed as nanograms/10^6 cells) were determined, as described previously (*26*). Cells were harvested from three independent cultures. Suspensions of ARPE-19 or D407 cells were centrifuged at 800*g* for 5 min at 4°C, and dissolved in 0.5 ml of 2 M oxalic acid. The mixtures were shaken vigorously and immediately heated for 30 min at 100°C. The mixtures without heating were used as a blank for measurement of endogenous porphyrins. After cooling down, fluorescence was determined with a RF-5300PC spectrofluorometer (Shimadzu Corp., Kyoto, Japan). The exciting light was at 400 nm, and the fluorescence emission was determined at 662 nm. Hemin solution was prepared in 0.5 ml oxalic acid, and used as the standard.

RESULTS

Hypoxia Decreases HO-1 Expression in HeLa Cells Despite HIF-1α Accumulation—We previously reported that the expression levels of HO-1 mRNA and protein were decreased in HeLa cervical cancer cells after 48 h incubation under hypoxia (*26*). To explore the role of HIF-1α for HO-1 expression, we analysed the expression levels of HO-1 mRNA together with HIF-1α protein under hypoxia in HeLa cells. The time-course study showed that the HO-1 mRNA level was decreased under hypoxia after 12 h (Fig. 1A). We also measured the level of HIF-1α protein under hypoxia by western blot analysis. Hypoxia led to the accumulation of HIF-1α protein, while the expression of HO-1 protein was reduced (Fig. 1B). In contrast, there was no noticeable change in the expression levels of HO-2 protein and β-actin

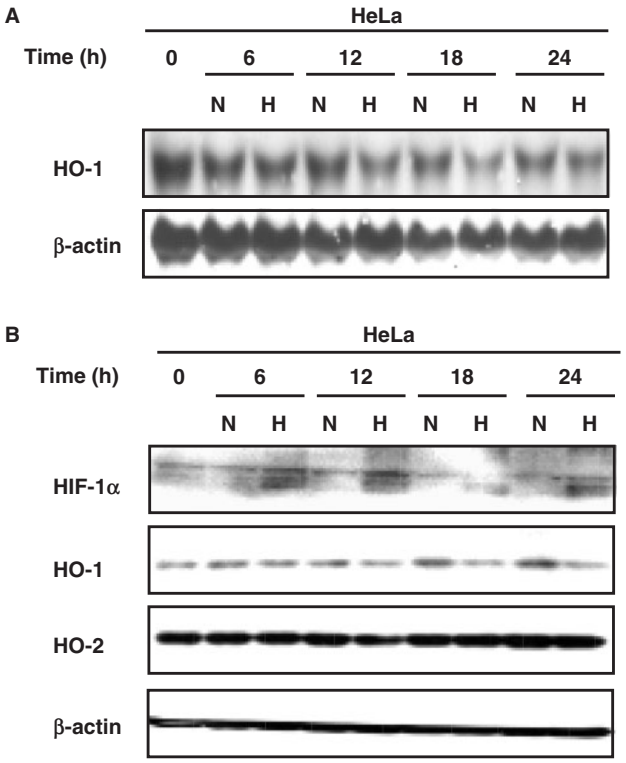

Fig. 1. **Effects of hypoxia on expression of HO-1 mRNA and protein in HeLa cells.** HeLa cells were cultivated under normoxia (20% O_2; N) or hypoxia (1% O_2; H) for the indicated time (h) and then harvested for RNA and protein preparation. Shown are representative examples of northern blot analysis (A) and western blot analysis (B). The data shown are from one of two independent experiments with similar results. (A) Northern blot analysis. Each lane contained 10 μg total RNA. The lane labelled with '0' contained RNA prepared from the untreated cells. A bottom panel shows β-actin as an internal control. (B) Western blot analysis. Each lane contained cell extracts (40 μg protein). The lane labelled with '0' contained protein prepared from the untreated cells. A bottom panel shows β-actin as an internal control.

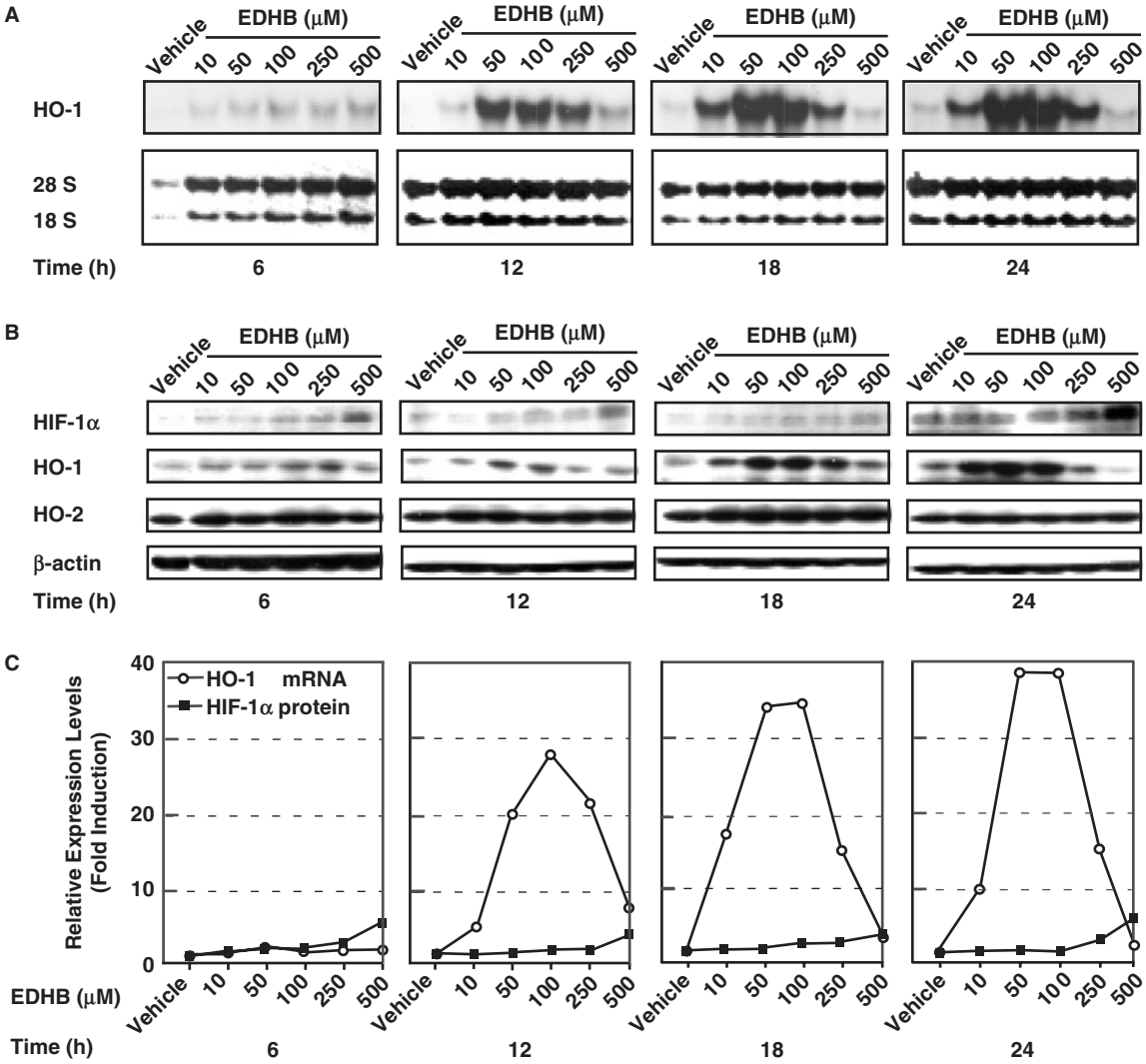

Fig. 2. **Time-course and dose–response of HO-1 expression with EDHB treatment in HeLa cells.** HeLa cells were treated with EDHB for the indicated time (h) and then harvested for RNA and protein preparation. (A) Northern blot analysis. Each lane contained 10 μg total RNA. The lane labelled with 'vehicle' contained RNA prepared from ethanol-treated cells. A bottom panel shows rRNAs as loading control (shown in reversed black and white). The data shown are from one of the two independent experiments with similar results. (B) Western blot analysis. Each lane contained cell extracts (40 μg protein). The lane labelled with 'vehicle' contained protein prepared from ethanol-treated cells. A bottom panel shows β-actin as an internal control. The data shown are from one of the two independent experiments with similar results, except for HO-1 protein. The HO-1 protein data shown are the representative of three independent experiments. (C) Relative expression levels of HO-1 mRNA and HIF-1α protein. The intensities of the signals in (A) and (B) were quantified, and the intensities representing HO-1 mRNA and HIF-1α protein were normalized with the intensities of 18S and β-actin, respectively. The intensity representing HO-1 mRNA or HIF-1α protein in the cells treated with vehicle is considered to be 1. The data shown are average values of two independent experiments.

protein (an internal control) during 24 h incubation under hypoxia. Thus, the accumulation of HIF-1α protein is associated with the decrease in HO-1 protein under hypoxia in HeLa cells.

Induction of HO-1 Expression by EDHB is not Correlated to HIF-1α Accumulation in HeLa Cells—To further study the role of HIF-1α in the regulation of HO-1 expression in HeLa cells, we analysed the effect of EDHB, a prolyl-hydroxylase inhibitor, which is expected to accumulate HIF-1α protein under normoxia. The time-course and dose–response study showed that HO-1 mRNA expression was induced by >20-times in HeLa cells after treatment with EDHB (50 μM) (Fig. 2A). Likewise, the expression of HO-1 protein was significantly induced after 18 h incubation with EDHB (Fig. 2B); the magnitude of the increase in HO-1 protein levels achieved with 100 μM EDHB was 2.74 ± 0.26-times at 18 h and 3.46 ± 0.53-times at 24 h ($P < 0.01$). In contrast, EDHB at 250 or 500 μM rather decreased HO-1 mRNA expression (Fig. 2A), whereas the accumulation of HIF-1α was detected only with the higher concentrations (250 μM and 500 μM) of EDHB (Fig. 2B and C). The expression levels of HO-2 and β-actin proteins remained unchanged. Thus, the EDHB-mediated induction of HO-1

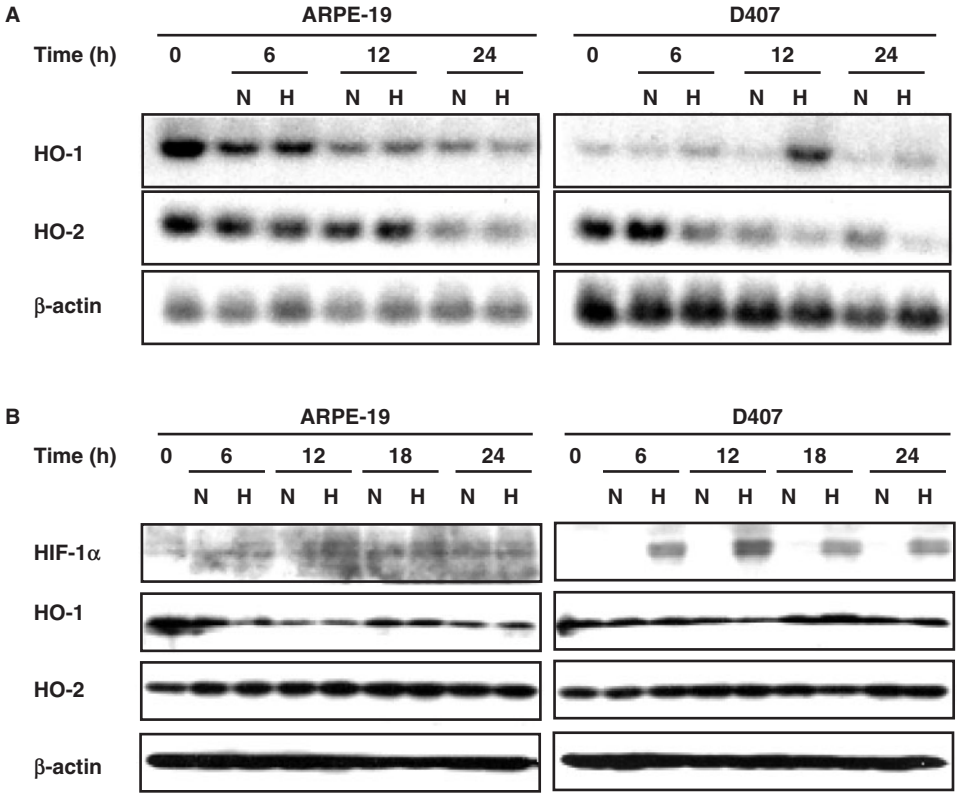

Fig. 3. **Effects of hypoxia on expression of HO-1 and HO-2 in human RPE cell lines.** ARPE-19 and D407 cells were cultivated under normoxia (20% O_2; N) or hypoxia (1% O_2; H) for the indicated time (h) and then harvested for RNA and protein preparation. Shown are representative examples of northern blot analysis (A) and western blot analysis (B). The data shown are from one of the two independent experiments with similar results. (A) Each lane contained 10 μg total RNA. The lane labelled with '0' contained RNA prepared from the untreated cells. (B) Each lane contained cell extracts (40 μg protein). The lane labelled with '0' contained protein prepared from the untreated cells. A bottom panel shows β-actin as an internal control.

expression is not correlated to HIF-1α accumulation in HeLa cells. Taken together, these results suggest that HO-1 expression may be regulated by a mechanism that is independent of HIF-1α.

Hypoxia Causes HIF-1α Accumulation in ARPE-19 and D407 RPE Cells—To explore the influence of hypoxia in RPE cells, we next used ARPE-19 and D407 human RPE cell lines, which showed the different responses to hypoxia in the expression of HO-1 mRNA; namely, hypoxia increased the expression level of HO-1 mRNA in D407 cells, but caused no noticeable change in ARPE-19 cells (*24*). Importantly, treatment with cycloheximide, an inhibitor for protein synthesis, prevented the hypoxia-mediated induction of HO-1 mRNA in D407 cells (*24*), suggesting the involvement of a newly synthesized protein factor, rather than HIF-1α, in the HO-1 induction. As expected, hypoxia induced the expression of HO-1 mRNA in D407 cells at 12 h, but not in ARPE-19 cells (Fig. 3A). It is also noteworthy that hypoxia reduced HO-2 mRNA expression in D407 cells, but not in ARPE-19 cells. Hypoxia increased the level of HIF-1α protein after 6 h of incubation in D407 cells, whereas it caused only marginal increase in the HIF-1α protein level in ARPE-19 cells (Fig. 3B). Moreover, there were no noticeable changes in the protein levels of HO-1, HO-2 and β-actin in both RPE cell lines. Thus, ARPE-19 and D407 RPE cells were able to respond to hypoxia by accumulating HIF-1α protein, with the different responses of HO-1 mRNA expression.

EDHB Induces HO-1 mRNA Expression in RPE Cells—We next explored the role of HIF-1α for HO-1 mRNA expression using EDHB. ARPE-19 cells were treated with EDHB and incubated for the indicated time (Fig. 4A). Northern blot analysis revealed that the expression of HO-1 mRNA was increased after the 12 h treatment with EDHB in ARPE-19 cells and remained at the induced levels for 24 h. The highest level of HO-1 mRNA was obtained with 100 μM EDHB. Western blot analysis revealed that HIF-1α protein was not significantly accumulated in ARPE-19 cells after treatment with EDHB even at 500 μM (Fig. 4B), while EDHB at 100 μM significantly increased HO-1 protein levels (4.23 ± 0.90-times at 18 h and 3.76 ± 0.9-times at 24 h, $P < 0.01$). The increase in HO-1 protein levels was preceded by the induction of HO-1 mRNA. There was no noticeable change in the protein levels of HO-2 and β-actin. Thus, EDHB may induce HO-1 expression in ARPE-19 cells through a mechanism that is independent of HIF-1α (Fig. 4C).

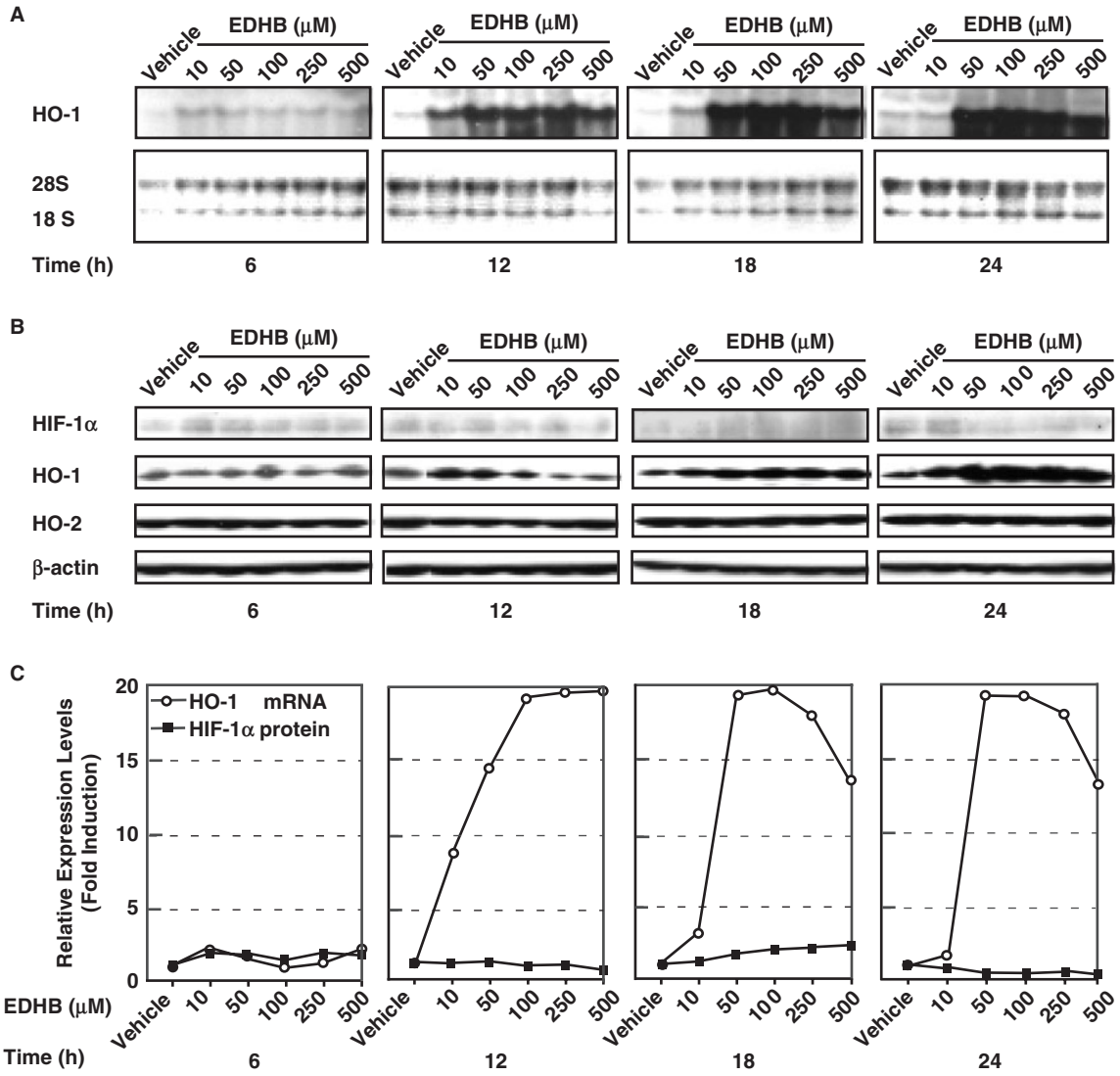

Fig. 4. **Time-course and dose–response of HO-1 expression with EDHB treatment in ARPE-19 cells**. ARPE-19 cells were treated with EDHB for the indicated time (h) and then harvested for RNA and protein preparation. Other conditions were the same as in Fig. 2. The data shown are from one of two independent experiments with similar results for northern blot analysis (A) and one of the three independent experiments with similar results for western blot analysis (B). Note that there was no statistical difference in the expression levels of HIF-1α protein. (C) Relative expression levels of HO-1 mRNA (average of two independent experiments) and HIF-1α protein (average of three independent experiments).

We also analysed the effects of EDHB in D407 RPE cells. EDHB at 50 μM caused the remarkable induction of HO-1 mRNA expression at 12 h (> 40-fold increase) in D407 RPE cells (Fig. 5A), but the highest concentration of EDHB (500 μM) rather decreased HO-1 mRNA level. In contrast, HIF-1α protein was accumulated in a dose-dependent manner in D407 cells (Fig. 5B), with the maximum accumulation of HIF-1α achieved with 500 μM EDHB. The increase in the expression of HO-1 protein was detected with 50 μM EDHB at 12 h, but the maximum induction was achieved with 100 μM EDHB (4.13 ± 0.95-times at 18 h and 3.71 ± 1.03-times at 24 h, $P < 0.01$). HO-2 and β-actin proteins remained unchanged in D407 cells. Thus, there was the difference in the sensitivity to EDHB between the induction of HO-1 mRNA expression and the HIF-1α protein accumulation (Fig. 5C). These results also suggest that EDHB may induce HO-1 expression in the HIF-1α-independent manner.

EDHB Activates the HO-1 Promoter in ARPE-19 Cells—To study the mechanism, by which EDHB increases the expression level of HO-1 mRNA, we next performed transient expression assays of the HO-1 gene promoter constructs in ARPE-19 cells. The basal promoter activity of phHOLUC45, which contains the MARE, was higher (26.54 ± 1.46-times) than the promoter activity of phHOLUC40, lacking the MARE (Fig. 6A). The basal promoter activity of phHOLUC40 was similar to that of phHOLUC-58. Thus, the upstream region (−4.5 kb and −4.0 kb) functions as an enhancer in ARPE-19 cells. We analysed the effect of EDHB (100 μM and 500 μM) on the HO-1 gene promoter in ARPE-19 cells.

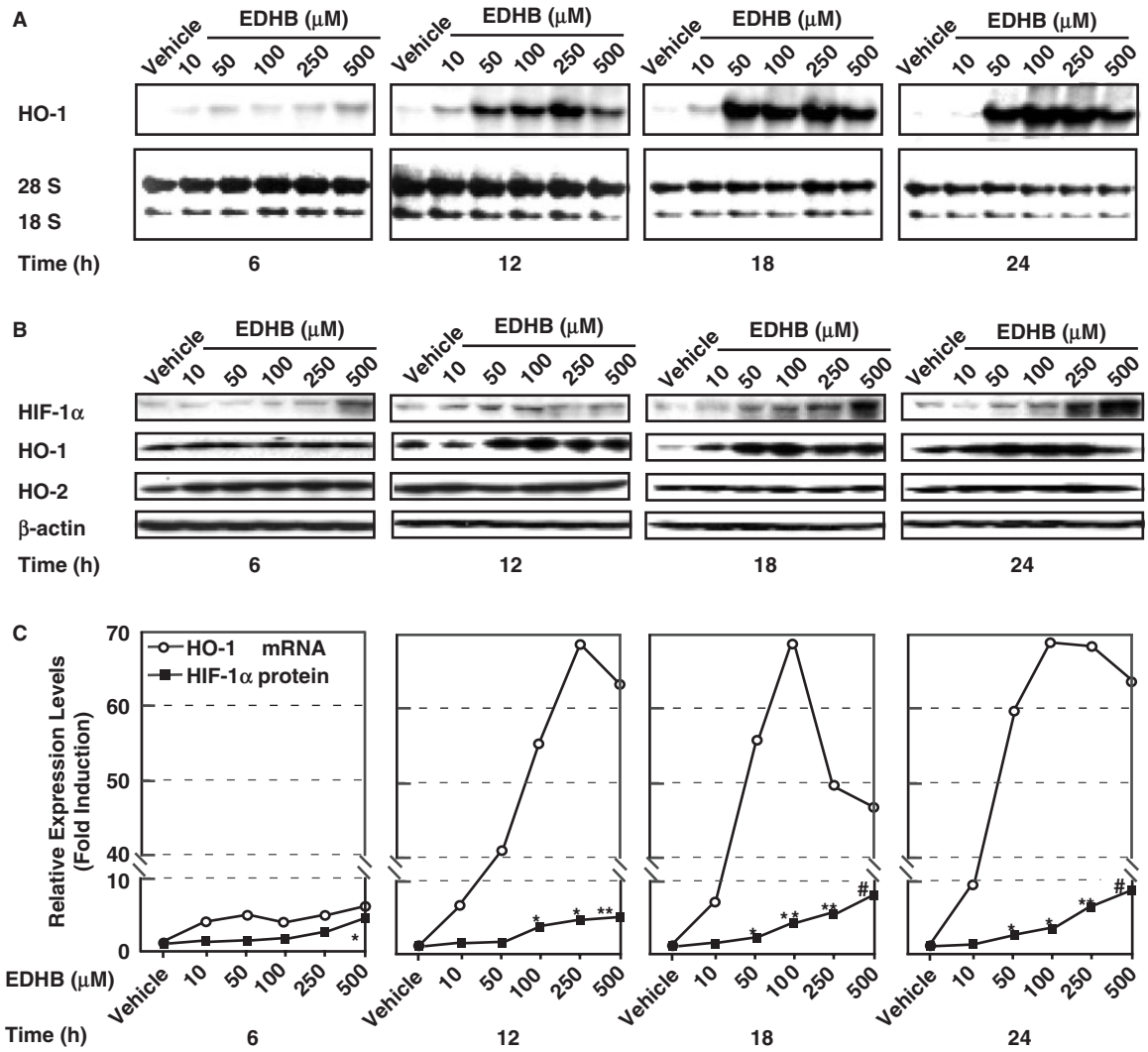

Fig. 5. **Time-course and dose–response of HO-1 expression with EDHB treatment in D407 cells.** D407 cells were treated with EDHB for the indicated time (h) and then harvested for RNA and protein preparation. Other conditions were the same as in Fig. 2. The data shown are from one of the two independent experiments with similar results for northern blot analysis (A) and one of the three independent experiments with similar results for western blot analysis (B). (C) Relative expression levels of HO-1 mRNA and HIF-1α protein. The data of HIF-1α protein are means of three independent experiments (each SD is not shown because of the limited space and small number). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.001$.

EDHB at 100 μM increased the promoter activity of phHOLUC45 (3.70 ± 0.19-times, $P < 0.001$), compared to the activity in transfected cells treated with ethanol, a vehicle. In contrast, EDHB showed no noticeable effects on the promoter activity of phHOLUC40 or phHOLUC-58, each of which lacks the MARE. These results suggest that EDHB may activate transcription of the HO-1 gene through the enhancer sequence, containing MARE. It is also noteworthy that the identified enhancer region (−4.5 kb and −4.0 kb) lacks a HRE, a binding site for HIF-1α.

To further explore the role of MARE for the EDHB-mediated increase in the HO-1 promoter activity, we used a model construct pRBGP2, which contains three copies of MARE. EDHB at either 100 μM or 500 μM significantly increased the promoter activity of pRBGP2 but not pRBGP4, containing three copies of the mutated MARE (Fig. 6B). On the other hand, EDHB even at 500 μM exerted no significant effect on the expression levels of HRESV40, which carries four copies of HRE, the binding site for HIF-1α, which is consistent with the results that EDHB treatment did not accumulate HIF-1α protein in ARPE-19 cells (Fig. 4B). It should be noted that hypoxia significantly increased the promoter activity of HRESV40 after 18 h incubation under hypoxia (5.69 ± 1.22-times, compared to the promoter activity under normoxia, $P < 0.01$), thereby confirming the validity of the transient expression assays. In contrast, hypoxia did not influence the promoter activity of phHOLUC45 in ARPE-19 cells (data not shown).

Differential Dose-dependent Effect of EDHB on the HO-1 Promoter and HRE in HeLa and D407 Cells—To obtain further evidence for the involvement of MARE in the EDHB-mediated induction of HO-1 mRNA

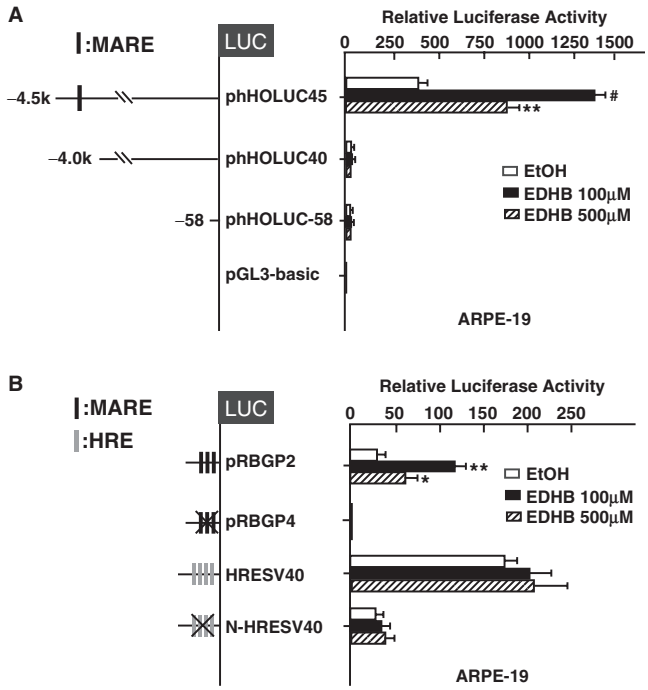

Fig. 6. **Effect of EDHB on the human HO-1 gene promoter activity in ARPE-19 cells.** ARPE-19 cells were transiently transfected with each reporter construct and were treated with EDHB at a final concentration of 100 μM or 500 μM for 18 h. (A) Promoter activities of HO-1 gene constructs. The parent construct phHOLUC45 contains the MARE. The constructs phHOLUC40 and phHOLUC-58 lack the MARE. A promoterless construct (pGL3-basic) was used as a control. (B) The test promoters pRBGP2 and pRBGP4 represent positive and negative controls for MARE activity. The two constructs, named HRESV40 and N-HRESV40, represent positive and negative controls for HRE. Shown is the ratio to the normalized luciferase activity of pGL3-basic (A) or pRBGP4 (B) in transfected cells treated with ethanol. The data are means ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.001$.

expression, we conducted transient expression assays in HeLa and D407 cells, in which HIF-1α protein was accumulated after treatment with 500 μM EDHB for 18 h (Figs 2 and 5). In HeLa cells, EDHB at 100 μM increased the promoter activity of phHOLUC45 (7.03 ± 1.13-times, $P < 0.001$), compared to the activity in transfected cells treated with ethanol, but 500 μM EDHB showed no noticeable effect on the promoter activity of phHOLUC45 (Fig. 7A). These results were consistent with the differential effects of EDHB at 100 and 500 μM on HO-1 mRNA induction in HeLa cells (Fig. 2A). In contrast, EDHB showed no noticeable effect on the promoter activities of phHOLUC40 or phHOLUC-58, lacking the MARE. On the other hand, in D407 cells, EDHB at 100 and 500 μM increased the promoter activity of phHOLUC45 (1.99 ± 0.12-times, $P < 0.01$ and 1.60 ± 0.09-times, $P < 0.05$, respectively), compared to the activity in control cells (Fig. 7A). In contrast, hypoxia did not influence the promoter activity of phHOLUC45 in D407 cells (*24*).

EDHB at 100 μM, but not at 500 μM, increased the promoter activity of pRBGP2 in HeLa cells (5.33 ± 0.70-times, $P < 0.001$), whereas EDHB at either 100 μM (2.14 ± 0.73-times, $P < 0.05$) or 500 μM (1.82 ± 0.37-times, $P < 0.05$) increased the promoter activity of pRBGP2 in D407 cells (Fig. 7B). Moreover, EDHB at 500 μM significantly increased the promoter activity of HRESV40 in both D407 cells (2.54 ± 0.62-times, $P < 0.05$) and HeLa cells (2.55 ± 0.10-times, $P < 0.05$), but EDHB at 100 μM exerted no noticeable effect on the promoter activity of HRESV40. These results are in good agreement with the accumulation of HIF-1α protein in both cell lines observed after treatment with 500 μM EDHB. These results also suggest the different susceptibilities of MARE and HRE to EDHB. Taken together, EDHB may induce HO-1 expression in the HIF-1α-independent manner.

Effect of EDHB on the Expression of HO-1 mRNA in A549 Cells—To study the role of MARE for the EDHB-mediated HO-1 induction, we next used A549 human lung cancer cells, in which HO-1 mRNA is overexpressed due to the sustained activation of Nrf2, a transcriptional activator, caused by the mutation of the Keap1 gene (*37*). It has been reported that Nrf2 activates HO-1 gene transcription by binding to MARE (*38*). HO-1 mRNA expression was increased about 6-folds after 12 h incubation with EDHB at 100 μM (Fig. 8A), whereas HO-1 protein levels remained unchanged (Fig. 8B). HIF-1α was rapidly accumulated after EDHB treatment (Fig. 8B). There was no noticeable change in the protein levels of HO-2 and β-actin. Thus, EDHB treatment increased HO-1 mRNA expression in A549 cells, the magnitude of which was however lower than that seen in other cell lines (Fig. 8C). Moreover, the induction of HO-1 mRNA expression was not paralleled to HIF-1α protein accumulation.

DISCUSSION

The RPE may encounter hypoxia under serious pathological conditions, such as retinal vascular occlusive diseases and retinal detachment, in which angiogenesis is eventually enhanced, as seen in diabetic retinopathy and age-related macular degeneration. Accordingly, retinal hypoxia may be followed by neovascularization, which is normally suppressed in the retina. In the present study, we have shown that hypoxia causes the accumulation of HIF-1α protein in D407 and ARPE-19 RPE cells as well as in HeLa cervical cancer cells, although the degree of accumulation was only marginal in ARPE-19 cells. Moreover, hypoxia exerted the differential effects on the expression of HO-1 mRNA in these cell lines; namely, HO-1 mRNA expression under hypoxia was increased in D407 cells, remained unchanged in ARPE-19 cells (*24*) and decreased in HeLa cells (*26*). These results indicate that expression of HO-1 mRNA is not directly regulated under hypoxia by HIF-1α. It is also noteworthy that ARPE-19 cells are characterized by the lack of HIF-1α protein accumulation after treatment with EDHB, a prolyl-hydroxylase inhibitor, whereas EDHB caused HIF-1α accumulation in D407 cells. Thus, ARPE-19 cells are less sensitive to hypoxia and EDHB, compared with D407 cells, with respect to HIF-1α accumulation, suggesting that the expression of HIF-1α protein may be regulated in ARPE-19 cells in a manner

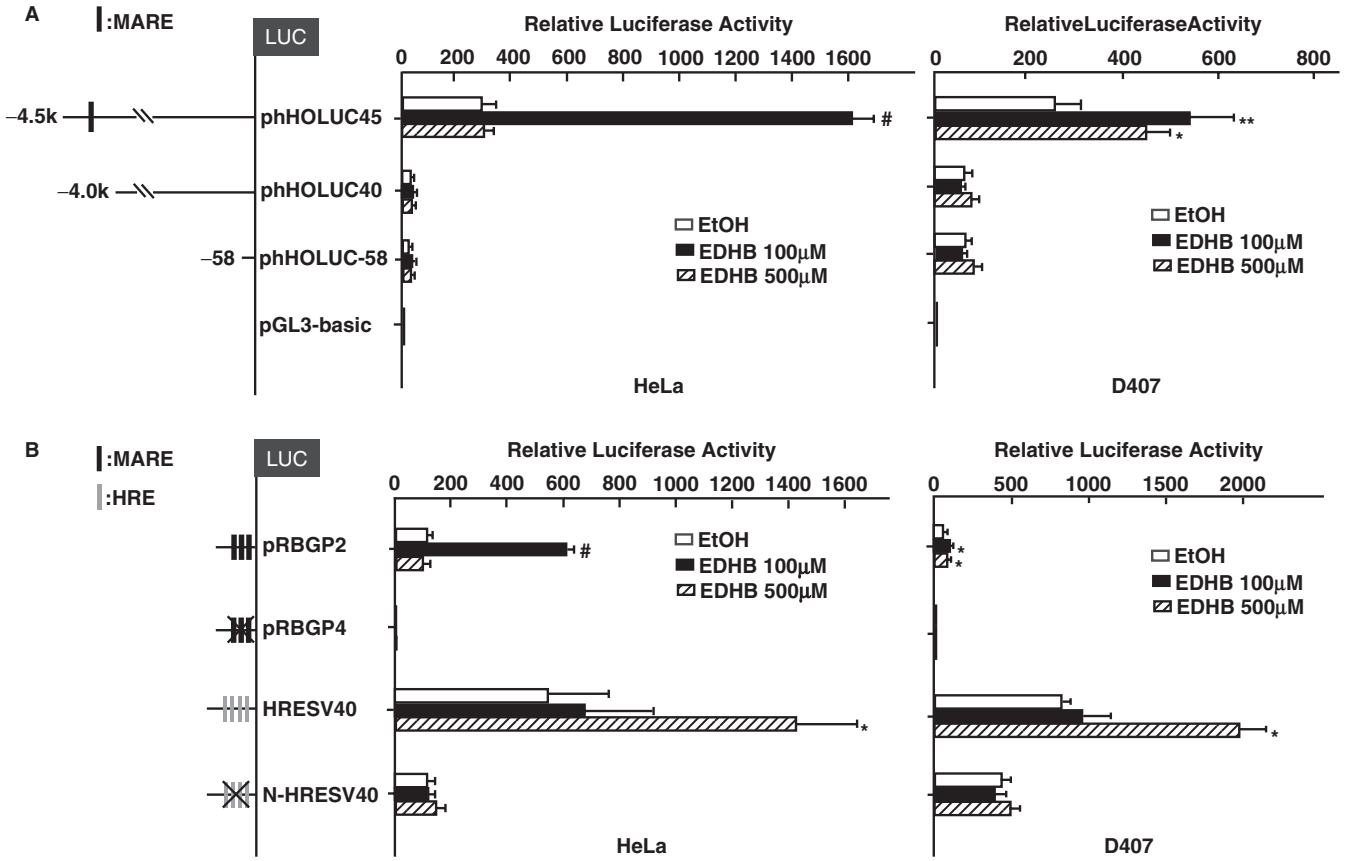

Fig. 7. **Effect of EDHB on the human HO-1 gene promoter activity in HeLa and D407 cells.** HeLa and D407 cells were transiently transfected with each reporter construct and were treated for 18 h with EDHB at a final concentration of 100 μM or 500 μM. The data show the effect of EDHB on the HO-1 gene promoter (A) and test promoters (B). The constructs used were the same as in Fig. 6. Shown is the ratio to the normalized luciferase activity of pGL3-basic (A) or pRBGP4 (B) in transfected cells treated with ethanol. The data are means ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.001$.

different from that in D407 cells. In this context, ARPE-19 cells appear to preserve the properties of differentiated RPE (*29*, *39*), in which the blunted accumulation of HIF-1α protein may be beneficial for retinal homeostasis. Such a difference in the regulation of HIF-1α protein expression may reflect the regional variations in the differentiation state or proliferative capacity of RPE cells (*40*, *41*). In this context, Miyamura *et al*. (*42*) have reported a mosaic pattern of expression of HO-1 mRNA and protein in macular and peripheral RPE and the age-related decline in HO-1 mRNA expression in the macula and periphery. It is therefore conceivable that RPE may be characterized by the regional difference in the regulation of not only HO-1 but also HIF-1α.

It is also noteworthy that there was no noticeable change in the HO-1 protein level in D407 RPE cells under hypoxia, despite the induction of HO-1 mRNA expression (Fig. 3). In our previous report (*24*), however, we detected the marginal increase in HO-1 protein level in D407 cells under hypoxia. We therefore repeated the similar experiments in D407 cells and have concluded that hypoxia did not increase HO-1 protein level in D407 cells. We also measured the level of HO-2 protein, because HO-2 has been proposed to function as an oxygen sensor (*43–45*) and the expression of HO-2 protein was decreased under hypoxia in several human cell lines, including HeLa cells (*26*). It is noteworthy that in our earlier study, the HO-2 protein level was decreased in HeLa cells after 48 h incubation under hypoxia (26), while in this study we followed the short time-course (up to 24 h) to focus on the expression of HIF-1α protein. The expression level of HO-2 protein remained unchanged during 24 h incubation under hypoxia in ARPE-19 and D407 RPE cells (Fig. 3). Thus, the expression levels of both HO-1 and HO-2 proteins remained unchanged under hypoxia in ARPE-19 and D407 RPE cells, suggesting that haem catabolism may be maintained in the narrow range under hypoxia, despite the noticeable changes in HO-1 and HO-2 mRNA levels. In fact, we found that the haem content in ARPE-19 cells (1.70 ± 1.14 ng/10^6 cells) was significantly lower than that in D407 cells (19.97 ± 4.59 ng/10^6 cells; $P < 0.003$), and that treatment under hypoxia for 24 h did not noticeably change the haem contents in both RPE cell lines (data not shown). It is therefore conceivable that expression levels of HO-1 and HO-2 proteins may be regulated at the translational level and/or the post-translational level. Such a hitherto unknown mode of regulation would be important in the maintenance of cellular haem content in the RPE,

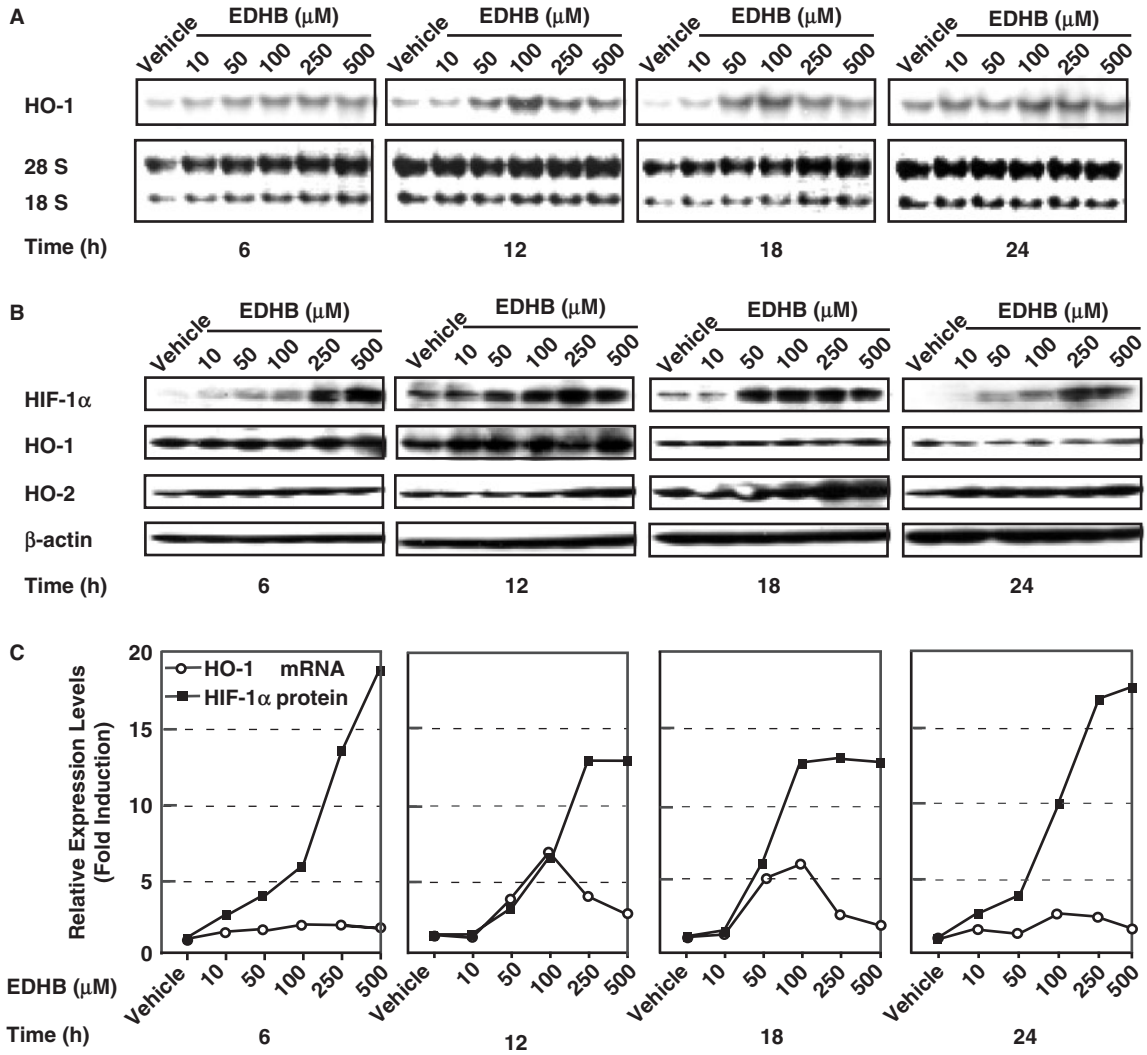

Fig. 8. **Time-course and dose–response of HO-1 expression with EDHB treatment in A549 cells.** A549 cells were treated with EDHB for the indicated time (h) and then harvested for RNA and protein preparation. Other conditions were the same as in Fig. 2. The data shown are from one of the two independent experiments with similar results: northern blot analysis (A) and western blot analysis (B). (C) Relative expression levels of HO-1 mRNA and HIF-1α protein. The data shown are average values of two independent experiments.

as haem is an essential molecule for all types of cells (*45*).

It has been reported that EDHB treatment induces HO-1 protein level in cultured mouse cardiomyocytes after 20 h incubation (*46*). Moreover, dimethyloxalylglycine, another prolyl-hydroxylase inhibitor, increased HO-1 protein levels in mouse cardiomyocytes (*46*) and human microvascular endothelial cells (*47*). Taken together with the present study, we suggest that EDHB may elicit a certain metabolic change, which in turn induces HO-1 expression. Indeed, we have provided evidence that EDHB may induce HO-1 mRNA expression by acting on the HO-1 gene enhancer. The relevant enhancer is located about 4.0 kb upstream from the transcription initiation site of the human HO-1 gene, and contains MARE that is recognized by the NF-E2-related factor-2 (Nrf2) (*48*, *49*). Nrf2 is sequestered in the cytoplasm as an inactive complex with its cytosolic repressor Keap1 (*50*). Dissociation of Nrf2 from the inhibitory protein Keap1 is a prerequisite for nuclear translocation of Nrf2. In A549 cells, carrying a Keap1 mis-sense mutation, the degree of EDHB-mediated induction of HO-1 mRNA is lowest (only 6-fold induction) among the cell lines examined in the present study. It is therefore conceivable that EDHB may induce HO-1 gene expression at least in part through Nrf2.

The present study has also shown the inter-cell difference in the sensitivity to EDHB, with respect to the MARE-mediated activation and the HRE-mediated activation of the reporter genes. Treatment with EDHB at 100 μM increased the expression of the HO-1 construct carrying MARE both in ARPE-19 and D407 cells, but did not influence the expression of the HRE test construct. On the other hand, treatment with EDHB at 500 μM increased the HRE test promoter activity in D407 cells, but not in ARPE-19 cells, which is consistent with the

results that 500 μM EDHB increased HIF-1α protein in D407 cells, but not in ARPE-19 cells. In fact, 500 μM EDHB rather decreased the expression of HO-1 in RPE cell lines. This is the first report, showing the dose-dependent differential effects of EDHB on HO-1 and HIF-1α expression.

In summary, we have provided evidence that the expression of HO-1 mRNA is regulated in RPE cells under hypoxia through a mechanism that is independent of HIF-1α. Treatment with EDHB consistently induces HO-1 expression in the human cell lines examined, which is likely to be mediated by the MARE. EDHB should be cautiously used to explore a role for HIF-1α.

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CONFLICT OF INTEREST

None declared.

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