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Research Article

Activation of hypoxia-inducible factor-1 regulates human histidine decarboxylase expression

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Abstract. Histidine decarboxylase (HDC) catalyzes the formation of histamine from histidine. Histamine has various effects in physiological and pathological reactions, such as inflammation, cell growth, and neuro-transmission. We investigated the role of hypoxia-inducible factor (HIF)-1 on hypoxia-induced HDC expression in human mast cell line, HMC-1 cells and mouse bone marrow-derived mast cells (BMMCs). Hypoxia significantly increased histamine production. HDC expression and activity were induced by hypoxia. Additionally, when cells were transfected with a native form of HIF-1α, hypoxia could induce higher HDC expression than in the non-transfected cell. HIF-1 binding activity for HDC 5' flanking region (HFR) was similar to that for the hypoxia-responsive element. Using HDC promoter deletion analysis, we also demonstrated that HFR was regulated by HIF-1 activation. In addition, depletion of HIF-1α prevents hypoxic induction of HDC in BMMCs. In conclusion, these results demonstrate that hypoxia induces HDC expression by transcriptional mechanisms dependent upon HIF-1.

Keywords. Histidine decarboxylase, hypoxia, mast cell, hypoxia inducible factor-1, histamine.

Introduction

Hypoxia is an essential developmental and physiological stimulus that plays a key role in the pathophysiology of heart disease, cancer, neuron death, cerebrovascular disease, and chronic lung disease, which represent the most common causes of mortality in western cultures [1].

Histamine plays an important role in a wide variety of reactions, such as the allergic reaction, inflammation, gastric acid secretion, neurotransmission in the central nervous system (CNS), and cell growth [2]. Basophils and mast cells are the main sources of histamine, which is formed from L-histidine by histidine decarboxylase (HDC) [3]. Mast cells are widely distributed in the connective tissues of mammals and other

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vertebrates, where they are frequently located in close proximity to blood vessels [4]. In chronically hypoxic rats, histamine-containing mast cells are increased and the increased mast cells contribute to a significant extent to acute hypoxia-induced injury [5]. But, it has remained unknown whether hypoxia induces the migration of histamine-containing mast cells or induces the synthesis of histamine in mast cells. Furthermore, roles and synthetic mechanisms of histamine have not been well defined yet for the hypoxic condition.

Recent studies have indicated that hypoxia-inducible factor 1 (HIF-1) is produced or activated in response to hypoxia [6]. HIF-1 is expressed in response to hypoxia in most cell types and activates the transcription of genes encoding proteins that function to increase O_2 delivery, allowing metabolic adaptation, increasing inflammation or promoting cell survival under hypoxic or ischemic conditions [7]. HIF-1 consists of HIF-1α and HIF-1β subunits. HIF-1β can dimerize with several different bHLH-PAS proteins, whereas HIF-1α is the specific and O_2-regulated subunit of HIF-1 that determines its biological activity. The heterodimer can then bind to hypoxic response elements (HRE) in the above-mentioned gene and increase their expression [8].

In this report, we tried to elucidate the role of HIF-1α on hypoxia-induced HDC expression in mast cells.

Materials and methods

Cell culture. Mast cell line HMC-1 was used in this study. Cells were maintained in IMDM medium (Gibco BRL, USA) with 10% fetal bovine serum (JRH BIOSCIENCE, USA) at 37 °C under 5% CO_2 in air. Mouse bone marrow-derived mast cells (BMMCs) were generated from the femoral bone marrow cells of female mice. Cells were incubated in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum or FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM 2-mercaptoethanol, 10 mM sodium pyruvate, 10 μM MEM nonessential amino acid solution (Invitrogen, Carlsbad, CA, USA), 100 U/ml murine IL-3 (R & D system Inc, Minneapolis, MN, USA), and 0.5 U/ml murine stem cell factor (R & D system Inc, Minneapolis, MN, USA) at 37 °C in a humidified atmosphere in the presence of 5% CO_2. After four to five weeks of culture, more than 96% of cells were identifiable as mast cells as determined by toluidine blue staining and fluorescence-activated cell sorting analysis of cell surface expression of c-Kit and FcεR I. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the US guidelines (Guide for the care and use of laboratory animals, 1985).

Hypoxia treatment. Cells were placed into an anaerobic chamber (O_2 tension $< 0.2\%$) (Anaerobic System 1025, Forma Scientific, Marietta, OH) and incubated at 37 °C within the chamber for varying durations.

Histamine assay. Histamine was measured from cells according to the manufacturer's specification using a histamine assay kit (R & D system Inc, Minneapolis, MN, USA).

Assay of VEGF production. VEGF production was measured by a modified ELISA method. The ELISA was devised by coating 96-well plates of monoclonal antibody with specificity for VEGF (R & D system Inc, Minneapolis, MN, USA). Before subsequent steps in the assay, coated plates were washed with phosphate-buffered saline (PBS) containing 0.05% tween-20 (PBST). All reagents used in this assay were incubated for 2 h at 37 °C. Recombinant VEGF was diluted and used as a standard. Serial dilutions starting at 5 ng/ml were used to establish the standard curve. Assay plates were exposed sequentially to biotinylated VEGF, and avidine peroxidase, and 2'-AZINO-bis (3-ethylbenzithiazoline -6-sulfonic acid) substrate solution containing 30% H_2O_2. The plates were read at 405 nm.

HDC Assay. Cells were suspended in an HDC reaction buffer of 0.1 M potassium phosphate buffer (pH 6.8) containing 0.2 mM DTT, 0.01 mM pyridoxal-5-phosphate, 1% polyethylene glycol ($M_r = 300$) and 100 μg/ml phenylmethylsulfonyl fluoride, sonicated for 20 s, and centrifuged at 10,000 × g for 15 min at 4 °C. Supernatant containing HDC protein was dialyzed overnight against HDC reaction buffer. After a brief centrifugation, an aliquot of the supernatant was incubated with 1 ml of HDC reaction buffer containing 0.25 mM L-histidine for 2 h at 37 °C. The reaction was stopped by adding 40 μl of 6.2 M perchloric acid. After a brief centrifugation, the histamine content of the supernatant was measured as described above. HDC activity was expressed as the amount of histamine formed per min per mg of protein. Protein was measured using bicinchoninic acid protein assay method.

RT-PCR analysis. Total RNA was isolated from cells according to the manufacturer's specification using easy-BLUE™ RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total

RNA (2.5 μg) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37°C using cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR was performed with the following primers for human (h) HDC (5'CAA GCA CAT GTC AGA CAT GG 3'; 5' TGA ACA GGA AGG AGG ACA GA 3'), hGAPDH (5'CAA AAG GGT CAT CAT CTC TG 3'; 5'CCT GCT TCA CCA CCT TCT TG 3'), mouse (m) HIF-1α (5' TGA GGC TCA CCA TCA GTT AT 3'; 5' TAA CCC CAT GTA TTT GTT C 3'), mHDC (5'GAT CAG ATT TCT ACC TGT GG 3';5' GTG TAC CAT CAT CCA CTT GG C 3'), and mGAPDH (5' TTC ACC ACC ATG GAG AAG GC 3'; 5' GGC ATG GAC TGT GGT CAT GA 3'). GAPDH was used to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 60 °C for hHDC, hGAPDH, and mHIF-1α, 50 °C for mHDC, and 62 °C for mGAPDH, respectively. Amplified fragment sizes for hHDC, hGAPDH, mHIF-1α, mHDC, and mGAPDH were 589 bp, 446 bp, 187 bp, 291 bp, and 236 bp, respectively. Products were electrophoresed on a 1.5 % agarose gel and visualized by staining with ethidium bromide.

Western blot analysis. Cell extracts were prepared by a detergent lysis procedure. Cells (5×10^6 cells) were scraped, washed once with PBS, and resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds every 15 min at 4 °C for 1 h and centrifuged at 15 000 × *g* for 5 min at 4 °C. Supernatants were assayed. Samples were heated at 95 °C for 5 min, and briefly cooled on ice. Following the centrifugation at 15 000 × g for 5 min, 50 μg aliquots were resolved by 10 % SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerin, and 20 % methanol at 25 V. Blots were blocked for at least 2 h with 1 × PBST containing 5 % nonfat dry milk. Protein was determined using a BCA (Sigma. St. Louis, MO, USA). The rabbit anti-HDC (Research Diagnostics, INC., Flanders, NJ, USA), and mouse anti-HIF-1α polyclonal antibody (Novus Biologicals, Littleton, CO, USA) were added and incubated for 1 h. Afterward, the nitrocellulose membrane was washed five times for 15 min with PBST. For protein detection, the blot was incubated with secondary antibody (1:3000 in PBST) conjugated with peroxidase for 40 min, followed by enhanced chemiluminesence detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Nuclear protein extraction. Preparation of crude nuclear extract was basically the same as described previously (26). Briefly, after cell activation for the times indicated, 5×10^6 cells were washed in 1 ml of ice-cold PBS, centrifuged at 1000 × *g* for 5 min, resuspended in 400 μl of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, O.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15 000 × *g* for 30 s. Pelleted nuclei were gently resuspended in 50 μl of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10 % glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15 000 × *g* for 5 min at 4 °C. Aliquots of the supernatant that contained nuclear proteins were frozen in liquid nitrogen and stored at -70 °C. Protein was determined using a BCA (Sigma. St. Louis, MO, USA).

Transient transfection and luciferase assay. For the transfection, we seeded the HMC-1 cells (1×10^7) in a 100 mm culture dish. We then used Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) to transiently transfect reporter gene constructs into HMC-1 cells. We mixed 20 μl of cell extract and 100 μl of the luciferase assay reagent at room temperature. To measure the luciferase activity, we used a luminometer (1420 luminescence counter, Perkin Elmer) in accordance with the manufacturer's protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of *firefly* luciferase activity to *renilla* luciferase activity

Transcription factor enzyme-linked immunoassay (TF-EIA). Avidine peroxidase was coated on 96-well ELISA plates. The coated plates were washed with PBST and then blocked with 3 % skim milk solution. The coated plates were incubated with 1 μg/ml of 5'-biotinylated single strand DNA oligonucleotide sequence for 1 h at room temperature. This sequence contains the HIF-1 binding motif, HIF-1 binding site wild type within the HDC 5' flanking region (HFR, the 5' flanking region of the human HDC gene from position -691 to position -655), or HIF-1 binding site mutant type within the HDC 5' flanking region (HFRM). The sequences used here were: 5'-ATCGCCCTACGTGCTGTCTCAGATC-3' for HIF-1 binding site (HRE), 5'-AGCA ACGT-GAAAACGTTGGATTACTTTTTACTCAGAA-3' for HFR, or 5'-AGCAAAAAG AAAACGTTG-GATTACT TTTTACTCAGA A-3' for HFRM. DNA binding reaction was carried out in a total volume 100 μl containing 1 μg nuclear protein extract in a buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 5 %

glycerol, 1 mM EDTA, and 1 mM DTT, for 1 h at room temperature and then washed. HIF-1α antibodies were then added at a 1:500 concentration in PBS containing 3 % BSA for 1 h, followed by the addition of the corresponding alkaline phosphatase-coupled secondary antibody. AP activity was then detected by the addition of p-nitrophenyl phosphate solution. After a 10 min incubation period, the reaction was arrested by the addition of 0.5 M H_2SO_4, Color intensity was detected at 405 nm using ELISA reader. AP activity was normalized to control values (unstimulated cells).

Statistical analysis of data. The experiments shown are a summary of the data from at least-three experiments and are presented as the mean ± SEM. Statistical evaluation of the results was performed by independent *t*-test and ANOVA with Tukey post hoc test. The results were considered significant at a value of $P < 0.05$.

Results

Hypoxia induces histamine synthesis and VEGF production. To address whether hypoxia induces histamine synthesis in HMC-1 cells, histamine assay was performed on supernatant and cell extract prepared from HMC-1 cells treated with medium, desferrioxamine (DFX, hypoxia mimic compound; 100 μM) or hypoxia for various times. As shown in Figure 1, DFX significantly enhanced histamine secretion and synthesis compared with the control ($P < 0.05$). Histamine secretion reached a peak 8 h after DFX treatment and histamine synthesis was continually increased up to 24 h after DFX treatment (Fig. 1A and B). Hypoxia also significantly increased the histamine secretion in a time-dependent manner (Fig. 1E). Previously, we reported that DFX induces HIF-1α activation [9]. To investigate the relations between HIF-1 and histamine synthesis, we studied the effect of iron (HIF-1 inhibitor) on DFX-induced histamine synthesis. We also treated disodium cromoglycate (DSCG) as an anti-histamine drug. The cells were pretreated with iron or DSCG for 1 h prior to DFX stimulation. Histamine content was significantly decreased by pretreatment of iron (about 59 %) or DSCG (about 63.6 %) (Fig. 1C). DFX or hypoxia also significantly increased the VEGF production (Fig. 1D and F, $P < 0.05$).

Hypoxia induces HDC expression and activation. To determine the effect of hypoxia on HDC expression and activation in mast cells, RT-PCR, Western blot analysis, and HDC assay were performed. Data as in Figure 2A, HDC mRNA levels were up-regulated in various times under DFX (100 μM) or hypoxia treatment. The expression of HDC increased up to 8 h after DFX treatment and declined thereafter from 12 to 24 h of DFX exposure. The hypoxia increased expression of HDC in a time-dependent manner (Fig. 2B). PMA+A23187 also increased HDC expression. The steady-state level of mRNA can be affected by the level of gene transcription and the stability of mRNA. To establish whether the increase in HDC mRNA levels after treatment with DFX takes place at the transcriptional level or the post-transcriptional level, we determined the effects of actinomycin D (AcD, 2.5 μg/ml) and cycloheximide (CHX, 20 ng/ml) on DFX-mediated HDC expression. AcD (RNA synthesis inhibitor) blocked this DFX-mediated up-regulation, whereas CHX (protein synthesis inhibitor) did not prevent the effect of DFX on HDC mRNA expression (Fig. 2C). HDC activity was also significantly increased by treatment of DFX ($P = 0.048$, Fig. 2D).

Inhibitory effect of iron or DSCG on the HDC expression and activation. To exclude the possibility of an inhibitory function of iron or DSCG, we analyzed HDC mRNA, protein, and activity levels directly by RT-PCR, Western blot analysis, and HDC assay. The cells were pretreated with ferrous iron or DSCG for 1 h prior to DFX stimulation for 4 h. As shown in Figure 3, addition of iron completely inhibited the DFX-induced HDC expression and activation ($P = 0.03$). DSCG had no effect on the HDC expression but significantly inhibited the HDC activity ($P = 0.02$).

Hypoxia induces the HIF-1α activation. We next examined whether DFX can modulate luciferase expression specifically via the HIF-1. The 3HRE-tk-LUC reporter was a kind gift from Dr Richard K. Bruick (University of Texas). Luciferase reporter gene constructs (3HRE-tk-LUC, plasmid containing HIF-1 binding site) were transiently transfected into HMC-1 cells, which was stimulated by DFX or PMA+A23187 for 24 h. PMA+A23187 used as a control (mast cell stimulator). As shown in Figure 4A, DFX or PMA+A23187 significantly increased reporter gene activity ($P < 0.05$). The HIF-1 activity induced by DFX and PMA+A23187 increased 13.3 and four-fold as compared with unstimulated cells, respectively. HIF-1 activation by DFX was higher than that by PMA+A23187. We next wanted to investigate whether HIF-1 protein levels induced by DFX could form active HIF-1 transcription complex. To perform these studies we performed a TF-EIA method. As shown in Figure 4B, DFX and PMA+A23187 increased DNA-

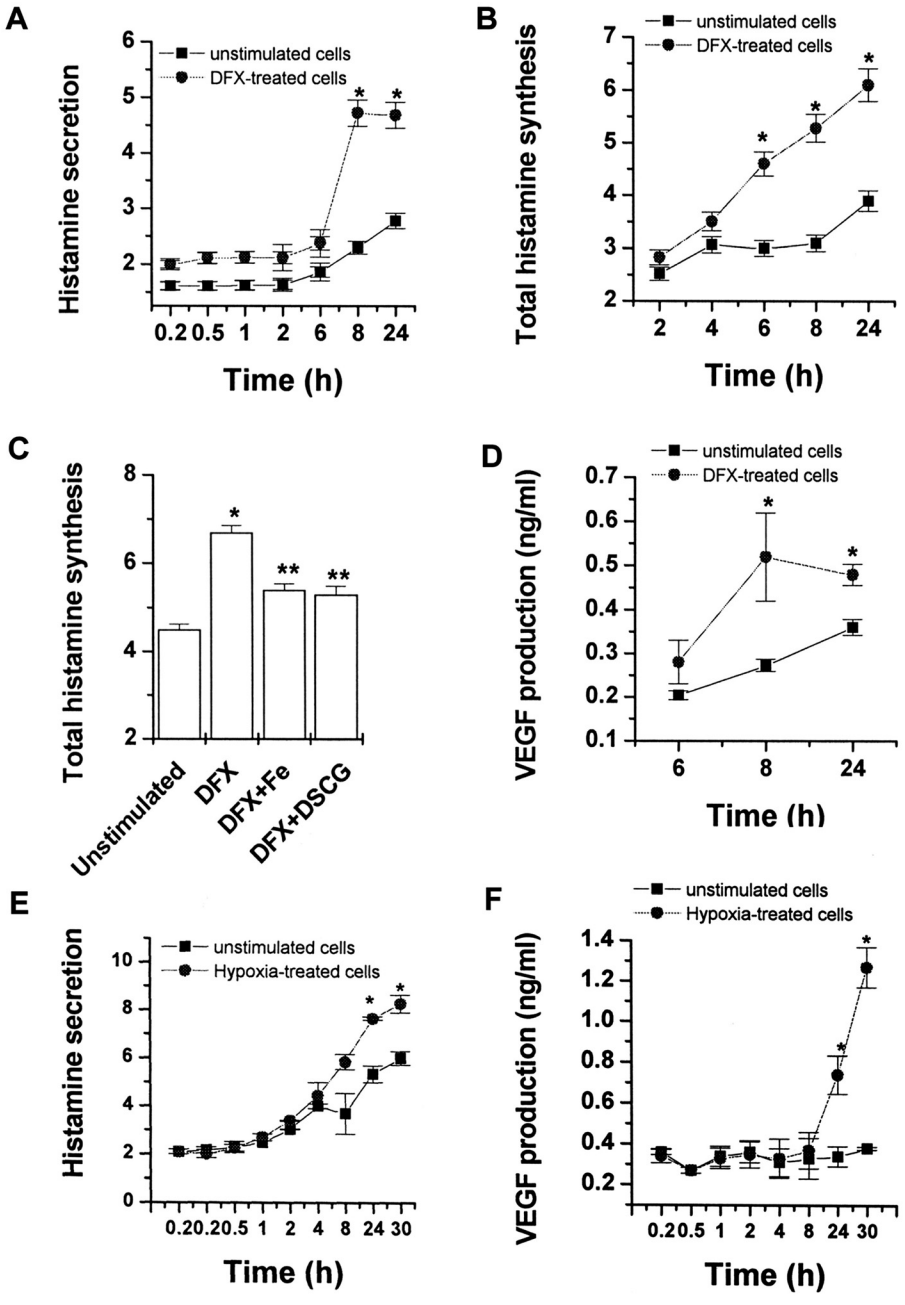

Figure 1. Hypoxia induces histamine synthesis and VEGF production. HMC-1 cells (5×10^5) were treated with DFX (100 μM) for various times. Secreted histamine (*A*) and total histamine (*B*) were assayed by using the histamine assay. HMC-1 cells were treated with iron (100 μM) or DSCG (10 μM) for 1 h and then stimulated with DFX (100 μM) for 24 h (*C*). VEGF concentrations were measured in cell supernatants using the ELISA (*D*). HMC-1 cells (1×10^6) were treated with hypoxia for various times. Secreted histamine (*E*) and VEGF (*F*) were assayed by using histamine assay and ELISA. *P < 0.05, significantly different from the unstimulated cells. ** P < 0.05, significantly different from DFX.

binding activity for HIF-1. This increased pattern is similar to Figure 4A results.

Role of HIF-1 in HDC expression by hypoxia in HMC-1 cells. We examined the influence of HIF-1 over-expression on HDC expression and activation during hypoxia. We used the plasmid pHAHIF-1 (wild type) and pCEP4/HIF-1αDN (mutant type). The protein expressed by pCEP4/HIF-1αDN consists of residues 1–3 of HIF-1α fused in-frame to residues 28–390 of HIF-1α and lacks the DNA binding and transactivation domains of the 826-ammino-acid wild type protein. HIF-1αDN competes with endogenous HIF-1α for dimerization with HIF-1β. But the HIF-1αDN/ HIF-1β complex neither binds to DNA nor activates transcription. Plasmids pHAHIF-1 or pCEP4/HIF-1αDN were transiently transfected into HMC-1 cells, the cells were stimulated by DFX for 4 h. HIF-1α. Plasmids pHAHIF-1 or pCEP4/HIF-1αDN were transiently transfected into HMC-1 cells, the cells were stimulated by DFX for 4 h. As shown in Figure 5, when cells were transiently transfected with pHAHIF-1, DFX significantly increased HDC expression and activation compared with un-transfected cells. In contrast, pCEP4/HIF-1αDN decreased HDC

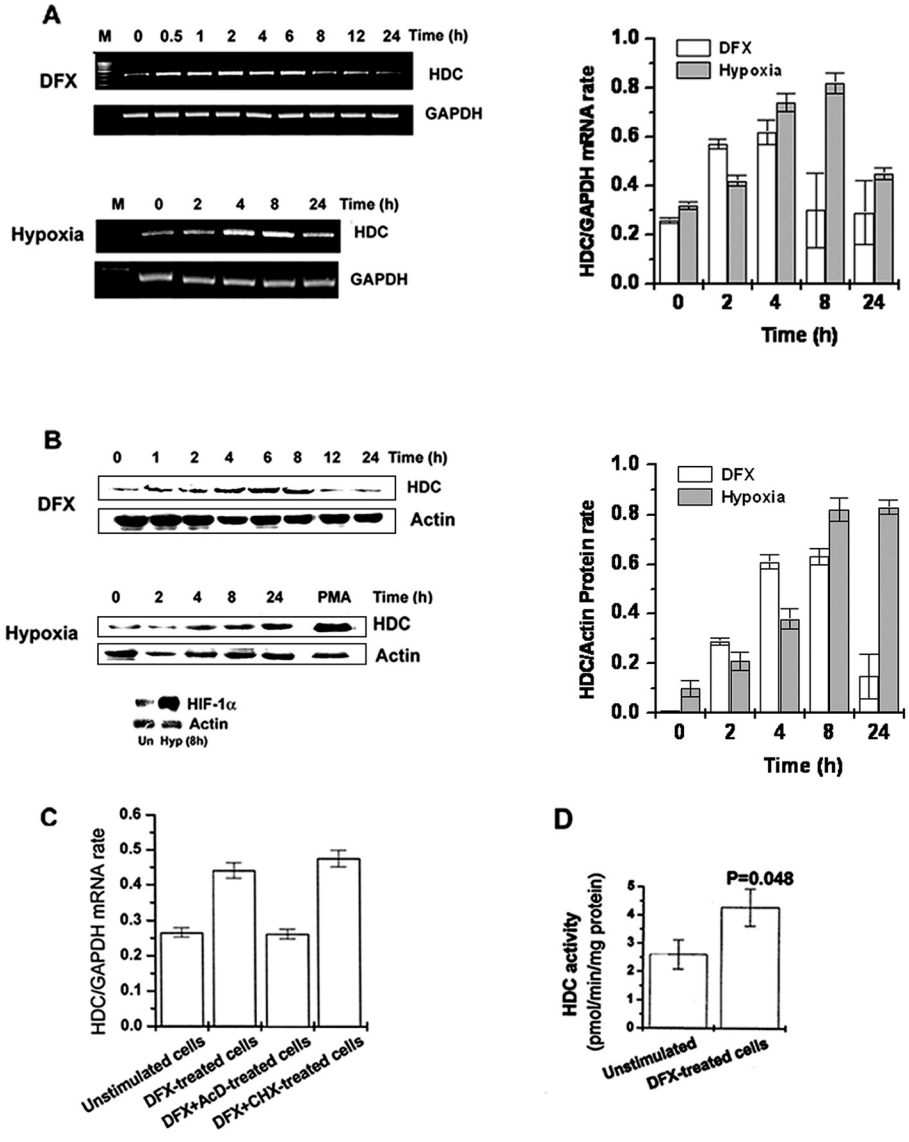

Figure 2. Hypoxia induces HDC expression and activation. HMC-1 cells (5×10^6) were treated with DFX (100 μM) or hypoxia for various times. The total RNA was assayed by RT-PCR analysis for HDC mRNA (*A*). The protein extracts were assayed by Western blot analysis for HDC (*B*). HMC-1 cells were treated with AcD (2.5 μg/ml) or CHX (20 ng/ml) for 1 h and then stimulated with DFX for 2 h (*C*). HDC activity was measured as described in Methods (*D*). Results are representative of three independent experiments, and the mean ± SEM of the band intensities correspond to the levels of HDC/GAPDH or Actin. M, marker; PMA, PMA+A23187; un, unstimulated cells; Hyp, hypoxia.

expression and activation compared with non-transfected cells.

Regulation of HDC 5' flanking region by HIF-1. We then evaluated whether the HIF-1 induced by DFX or hypoxia binds the HDC 5'flanking region. We performed the TF-EIA method. This assay uses HRE, HFR, and HFRM immobilized on a 96-well microplate. Protein complexes are then bound to the DNA oligonucleotide. As shown in Figure 6, HIF-1 binding activity for HRE (A) was significantly increased by treatment with DFX. It was similar to the binding activity for HFR (B). But HIF-1 binding activity for HFRM was not changed by treatment of DFX (data not shown) compared with unstimulated cells. HIF-1 binding activity for HRE under hypoxia was also similar to the binding activity for HFR (C and D). HIF-1 binding activity for HRE or HFR by DFX was higher than that by hypoxia.

Human HDC promoter is responsive to hypoxia. In order to study the effect of hypoxia stimulation on activity of human HDC promoter, 5'-deletion analysis was carried out on the original 1.8 kb HDC-luciferase construct; construct containing -125, -73, -59, and -47 bp of 5'-flanking DNA plus 126 bp of noncoding first exon were a kind gift from Dr. Wang (Columbia University). Plasmids were transiently transfected into HMC-1 cells, the cells were stimulated by hypoxia or DFX for 48 h. For each construct, the fold-increase in luciferase activity elicited by either hypoxia or DFX treatment was determined over basal luciferase activity from each promoter construct. As shown in Figure 6E, luciferase activities from cells transfected with 1.8 kb HDC-luciferase construct were significantly higher

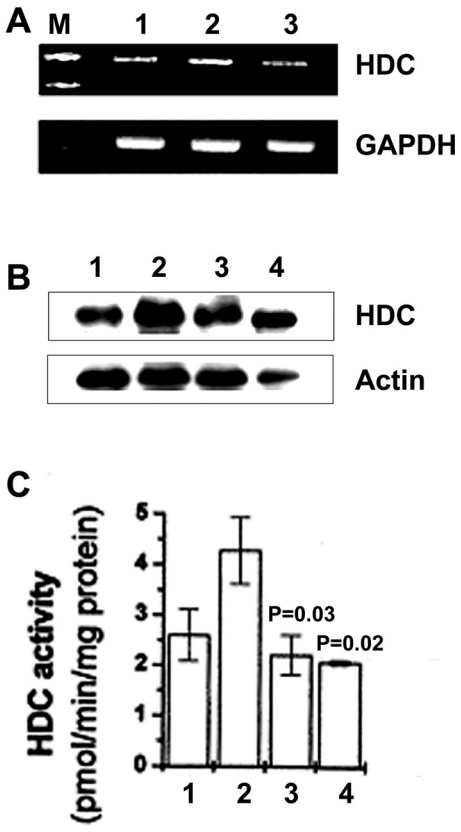

Figure 3. Inhibitory effect of iron or DSCG on the DFX-induced HDC expression and activation. HMC-1 cells (5×10^6) were treated with iron (100 μM), PDTC (10 μM), or DSCG (10 μM) for 1 h and then stimulated with DFX (100 μM) for 2 h. The total RNA was assayed by RT-PCR analysis for HDC mRNA (*A*). HMC-1 cells (5×10^6) were treated with iron (100 μM) or DSCG (10 μM) for 1 h and then stimulated with DFX (100 μM) for 4 h. Protein was prepared and analyzed for HDC expression (*B*) and activation (*C*). Results are representative of three independent experiments. M, marker; 1, unstimulated cells; 2, DFX; 3, DFX+iron (100 μM); 4, DFX+DSCG (10 μM).

than those transfected with -125, -73, -59, and -47 nucleotide constructs under hypoxia or DFX ($P < 0.05$).

Essential role of HIF-1α for HDC expression in BMMCs. In order to confirm whether up-regulation of HDC was mediated by HIF-1, we depleted HIF-1α in BMMCs using siRNA (HIF-1α SMARTpool, Dharmacon Inc, Chicago, IL, USA). Messenger RNA analysis by RT-PCR revealed HIF-1α was depleted. Western blot analysis was performed to confirm the depletion of HIF-1α at the protein level. As shown in Figure 7A, depletion of HIF-1α protein was achieved in BMMCs. HDC expression was also decreased by HIF-1α siRNA. Histamine secretion was inhibited by HIF-1α siRNA (Fig. 7B). Finally, to assess the functional effect of the knockdowns, the production levels of VEGF (HIF-1 target genes) were analyzed. Depletion of HIF-1α significantly reduced VEGF production (Fig. 7B).

Discussion

In the present study, we demonstrated for the first time that hypoxia induced histamine synthesis and HDC expression via activation of HIF-1 in mast cells. Histamine exerts a variety of proinflammatory and immunomodulating effects through interaction with four receptors, H1, H2, H3, and H4 [10, 11]. The immediate actions of histamine on vascular endothelium and on bronchial and vascular smooth muscle cells have been clearly elucidated. These effects are mostly mediated by the activation of the H1 receptor, and they are responsible for the majority of the acute symptoms in bronchial asthma, allergic rhinitis, and urticaria [12]. Histamine also exerts a variety of other regulatory functions by modulating and activity of T cells [13], monocytes [14], neutrophils [15], and eosinophils [16]. Moreover, high concentrations of histamine are found in areas of inflammation, which are mainly produced by mast cells [17]. Inflammatory lesions are characterized by hypoxia and dramatic recruitment of myeloid cells. Mast cells are located predominantly adjacent to capillaries and venules and are known to represent the major repository for histamine in various tissues [18]. Hypoxia resulted in rapid mast cell degranulation. Mast cell degranulation by hypoxia increases the reactive oxygen species, leukocyte adherence/emigration, and vascular permeability [5]. In this study, we found that histamine secretion could be increased by hypoxia. For that reason, we suggest that mast cells mediate the vascular inflammatory response to hypoxia.

Degranulation of mast cells can be triggered by at least two types of receptors: (1) high affinity IgE receptor, FcεRI, which is found on the plasma membrane of mast cells and basophils, and which may be transported to the membrane from intracellular stores of activated eosinophils, and (2) the G protein-coupled receptors found predominantly on mast cells, basophils, neutrophils and macrophages [5]. In this study, we show that hypoxia induced the degranulation of mast cells, such as in histamine release. Degranulation of mast cells was generally induced at an early stage. But, in this study, degranulation by hypoxia was delayed. Although the hypoxia leads to degranulation of mast cell, less is known of the signaling pathways that mediate degranulation by hypoxia. Further study is necessary to clarify the mechanism of the hypoxia-induced mast cell degranulation.

HDC is expressed in a variety of adult cell types, including mast cells/basophils, skin, platelets, hista-

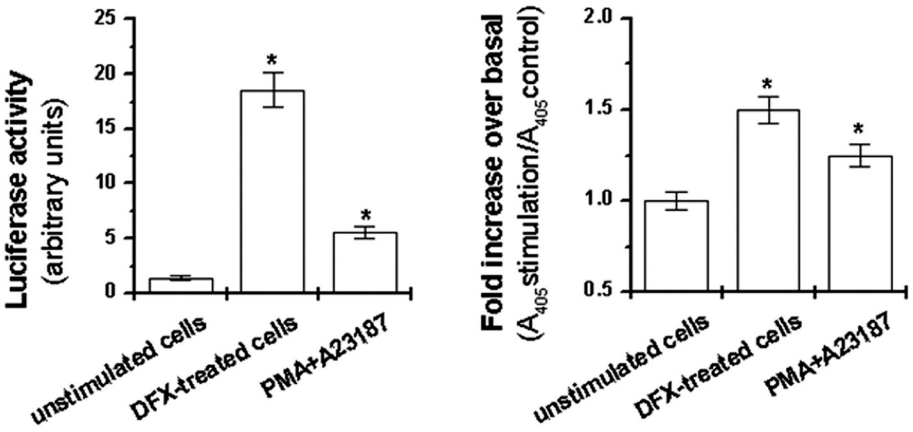

Figure 4. Hypoxia induces HIF-1α activation. HMC-1 cells (5×10^6) were transfected with 10 μg 3HRE-tk-LUC reporter construct. At 24 h after transfection, cells were stimulated with DFX for 24 h. At this point, HMC-1 cells were lysed and luciferase activity was measured using luciferase reporter assay system. Results are expressed as the ratio of firefly luciferase activity to renilla luciferase activity (*A*). HMC-1 cells (5×10^6) were stimulated with DFX. Nuclear protein (1 μg) was incubated in a 96-well plate coated with an oligonucleotide containing HRE binding site. Presence of HIF-1 transcription complex was evaluated with an antibody to HIF-1. HIF-1 binding was then revealed by incubation with an AP-conjugated secondary antibody and substrate. Results are expressed as the fold-increases of the absorbance at 405nm over control conditions (*B*). *P < 0.05, significantly different from the unstimulated cells.

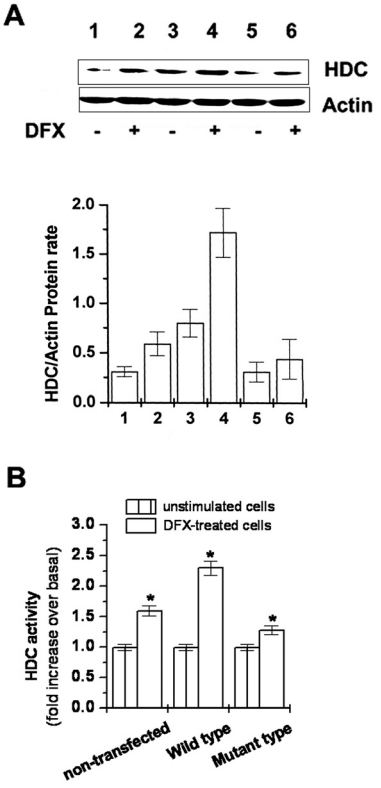

Figure 5. Role of HIF-1 in HDC expression by hypoxia. HMC-1 cells were transfected with 10 μg HIF-1 wild type and HIF-1 mutant type construct. At 24 h after transfection, cells were stimulated with DFX for 4 h. The protein extracts were assayed by Western blot analysis for HDC (*A*). HDC activity was measured as described in Methods (*B*). Results are representative of three independent experiments, and the mean ± SEM of the band intensities correspond to the levels of HDC/Actin. *P < 0.05, significantly different from the unstimulated cells. 1 and 2, non-transfected; 3 and 4, HIF-1α wild type transfected; 5 and 6, HIF-1α mutant type transfected.

minergic neurons in the brain, and enterochromaffin-like cells of the gastric corpus [19]. Yatsunami et al. reported that HDC is translated as a 74 kDa protein and is processed into a 54 kDa protein by releasing the carboxy-terminal in the human basophilic leukemia cell line, KU-812-F [20].

The same authors also reported that those two kinds of protein possess almost equivalent activity [20]. HDC mRNA and activity are constitutively expressed in KU-812-F cells. The levels of HDC mRNA and its enzymatic activity were reported to be increased by various stimulations such as PMA, dexamethasone, a combination of cyclic AMP and calcium ionophore A23187, and by chemically cross-linked oligomers of IgE in a few types of cells such as mouse mastocytoma P815, rat basophilic leukemia, and HMC-1 cells [21]. Tanaka et al. reported that a significant increase in HDC activity was first observed 2 h after the addition of anti-DNP-IgE, and a maximal level of HDC activity was obtained 6 h after the addition of anti-DNP IgE, and a significant increase in HDC mRNA expression was observed 1 h after stimulation with anti-DNP IgE. Cellular histamine content significantly increased at a later phase (12 h) with a fourfold increase in histamine content being detected 24 h after the addition of anti-DNP IgE [22]. In this study, total histamine content was continually increased up to 24 h (Fig. 1B) but expression of HDC was increased up to 8 h and declined thereafter from 12 to 24 h after DFX treatment (Fig. 2B). Data from Tanaka and his colleague basically agree with these findings.

The transcriptional regulation of HDC was induced by various transcription factors such as GATA, NF-E2, and Maf family protein MafK [23]. Transcriptional

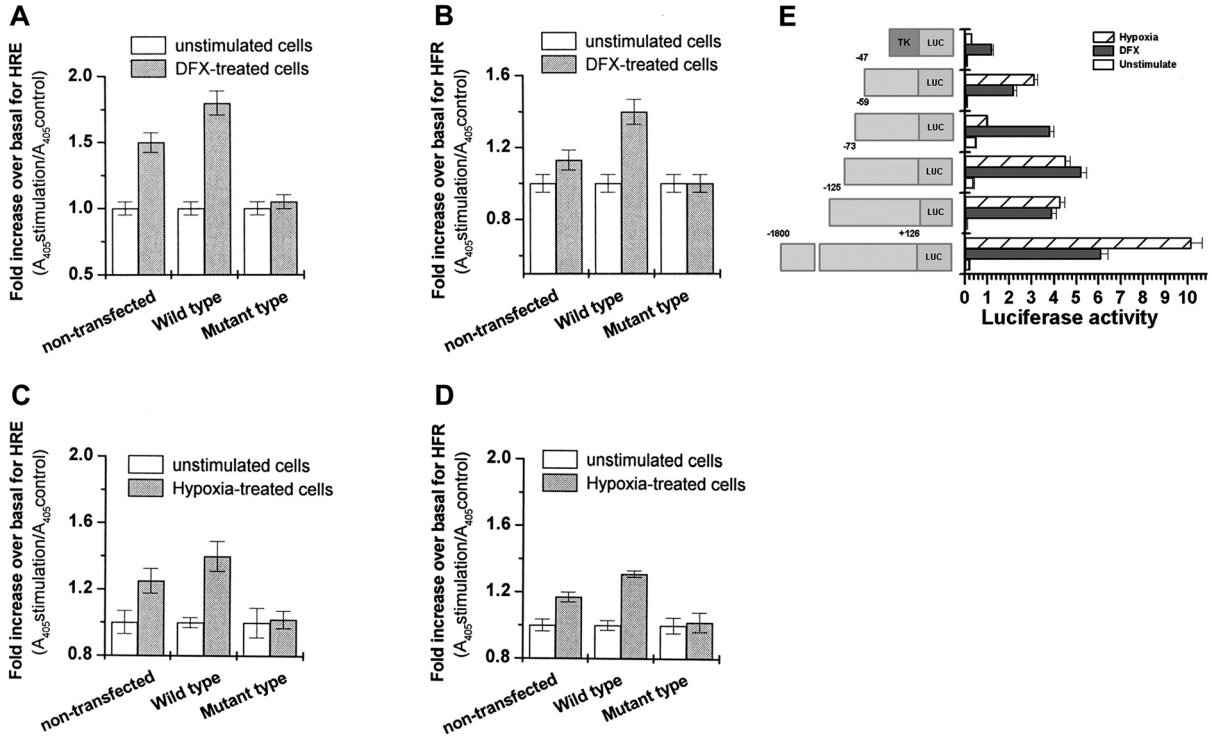

Figure 6. Regulation of HDC 5' flanking region by HIF-1. HMC-1 cells were transfected with 10 μg HIF-1 wild type and HIF-1 mutant type construct. At 24 h after transfection, cells were stimulated with DFX for 4 h (*A and B*) or hypoxia for 8 h (*C and D*). We performed the TF-EIA with nuclear protein (1 μg). Results are expressed as the fold-increases of the absorbance at 405nm over control conditions. 5'-deletion analysis of the human HDC promoter was carried out (*E*). Transfected HMC-1 cells were stimulated with DFX or hypoxia for 48 h and harvest, and luciferase activity was measured. Results are expressed as the ratio of firefly luciferase activity to renilla luciferase activity. *P < 0.05, significantly different from the unstimulated cells. TK Luc represents the activity of the construct pT81, which contains the enhancerless thymidine kinase promoter.

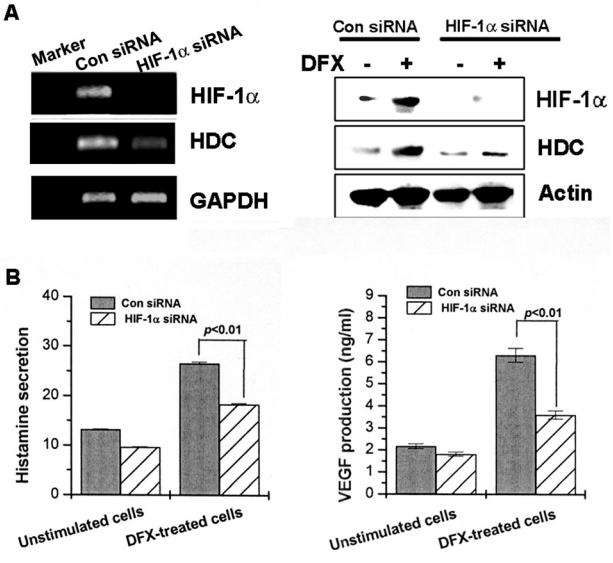

Figure 7. Essential role of HIF-1 for HDC expression of BMMCs. BMMCs were transfected with HIF-1α siRNA. BMMCs (5×10^6) were treated with DFX (100 μM). The total RNA was assayed by RT-PCR analysis for HIF-1α and HDC mRNA. The protein extracts were assayed by Western blot analysis for HIF-1α and HDC (*A*). Secreted histamine and VEGF were assayed by using histamine assay and ELISA (*B*). *P < 0.01, significantly different from the control siRNA transfected cells.

regulation of the HDC gene is also regulated by gastrin in the human gastric carcinoma cell line [19]. In this study, we found that hypoxia increased the HDC mRNA and protein expression, and HDC activity. AcD decreased the DFX-mediated HDC mRNA expression, whereas CHX did not, suggesting that the stimulation of HDC mRNA by DFX involves direct activation of the transcription of the HDC gene, and is independent of new protein synthesis. We also found that iron inhibited DFX-induced HDC expression and activation. DSCG inhibited the HDC activity but it did not affect HDC expression. From this, we suggested that HIF-1 functionally operates the DFX-induced HDC gene transcription.

The mechanisms for the regulation of gene expression under hypoxia are incompletely understood, but many researchers are recently beginning to report. Hypoxia regulates the various transcription factors such as nuclear factor-κB, activated protein-1, CCAAT/enhancer binding protein alpha, early growth response-1 and Ets-1 [9, 24]. Various transcription factors bind to the various conserved sequences on promoter region of genes, respectively. The positions of these conserved sequences differ from gene to gene. To further

elucidate the transcription regulation of HDC, we analyzed the luciferase expression of several HDC promoter constructs in response to both hypoxia and DFX. Both stimuli induced expression of luciferase activity in cells transfected with a luciferase construct containing the 1.8 kb of HDC promoter sequences. However, the hypoxic response is partially different from the response by DFX to luciferase activity of a -73 nucleotides construct. Riddle et al. demonstrated that the luciferase activity of hexokinase II promoter luciferase construct showed some differences in-between hypoxia and DFX [25]. Zhang et al. reported that human HDC is regulated by gastrin and PMA through a downstream cis-acting element [19]. HDC promoter activity by Gastrin or PMA is different from the activity in hypoxia. Therefore, we postulate that transcription factors activated by hypoxia are different from those activated by DFX except HIF-1. Further study is needed to fully characterize the potential consensus sequences of transcription factors in the HDC genes and to indentify the transcription factors activated by hypoxia and DFX.

In this study, depletion of HIF-1α by HIF-1α siRNA did not completely inhibit DFX-induced HDC expression. From this, we can presuppose that the endogenous HIF-1 partially plays a role in HDC expression.

Expression of HIF-1 was induced by hypoxic conditions, transition metals (Co^{2+}, Ni^{2+}, etc), nitric oxide, insulin, insulin-like growth factor, lipopolysaccharide, TNF-α, and dibenzoylmethane, etc. [26–28]. Under normoxia, HIF-1α is efficiently degraded by the proteasomal system, thus keeping its protein level extremely low [29]. Under hypoxia, the mRNA of HIF-1 appears unchanged, but HIF-1 protein accumulates, dimerizes with HIF-1 translocated to the nucleus, and binds to the target DNA sequence within HRE found in the promoter region of different genes [30]. Recent evidence has identified a link between inflammation, wound healing, and the activation of HIF-1 expression. Hollander et al. have demonstrated that HIF-1α protein was abundantly expressed by macrophages in inflamed rheumatoid synovia while being absent in healthy synovia [31]. Albina et al. reported that HIF-1α expression is strongly increased in inflammatory cells from wounds [32]. In a very elegant study, Cramer et al. demonstrated that a conditional knockout of HIF-1α in macrophages and other myeloid lineage cells leads to decreased myeloid cells infiltration and activation, to impaired chronic cutaneous inflammation, and to decreased joint inflammation in rheumatoid arthritis [33]. Taken together, these reports implicate HIF-1 as an important mediator of the inflammatory reaction.

Previously, we reported that DFX induced the production of proinflammatory cytokine through activation of HIF-1α [9]. Increased HIF-1α expression was decreased by iron [9]. In this study, we report that iron effectively inhibit the HDC expression and activity in DFX-stimulated HMC-1 cells. HDC expression was also decreased by transfection of HIF-1α siRNA on BMMCs. DFX increased the HIF-1 binding activity for HFR. HIF-1 binding activity for HRE was also similar to HIF-1 binding activity for HFR. When cells were transiently transfected with HIF-1α wild type plasmid (HIF-1α over-expression system), DFX increased HDC expression and activation compared with un-transfected cells. This study also demonstrates that 5'-flanking region of the human HDC is regulated by HIF-1 activation using an HDC promoter reporter gene assay. Luciferase activities from cells transfected with 1.8 kb HDC-luciferase construct were significantly higher than from those transfected with -125, -73, -59, and -47 nucleotide constructs under hypoxia. These results show that HIF-1 does bind to the HRE consensus sequence from -691 to -655 of HDC promoter under hypoxia. From this, we speculate that DFX increases the HDC expression and activity through activation of the HIF-1 under hypoxic conditions on mast cells. We propose that HIF-1 is a novel transcription factor of HDC gene.

In conclusion, our results imply that HDC expression is partially induced via evoking an HIF-1 response under hypoxic condition on mast cells. For that reason, the regulation of the HIF-1 pathway in mast cells may form the basis for a new strategy for the treatment of hypoxia-induced inflammatory diseases.

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