FEBS Letters 583 (2009) 55–60

FEBS Letters

journal homepage: www.FEBSLetters.org

Transcriptional activation of hypoxia-inducible factor-1α by HDAC4 and HDAC5 involves differential recruitment of p300 and FIH-1

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ARTICLE INFO

Article history:
Received 14 August 2008
Revised 21 October 2008
Accepted 22 November 2008
Available online 9 December 2008

Edited by Robert Barouki

Keywords:
HIF-1α
HDAC
FIH
p300

ABSTRACT

The interplay between hypoxia-inducible factor-1α (HIF-1α) and histone deacetylase (HDACs) have been well studied; however, the mechanism of cross-talk is unclear. Here, we investigated the roles of HDAC4 and HDAC5 in the regulation of HIF-1α function and its associated mechanisms. HDAC4 and HDAC5 enhanced transactivation by HIF-1α without stabilizing HIF-1α. HDAC4 and HDAC5 physically associated with HIF-1α through the inhibitory domain (ID) that is the binding site for factor inhibiting HIF-1 (FIH-1). In the presence of these HDACs, binding of HIF-1α to FIH-1 decreased, whereas binding to p300 increased. These results indicate that HDAC4 and HDAC5 increase the transactivation function of HIF-1α by promoting dissociation of HIF-1α from FIH-1 and association with p300.

Structured summary:
MINT-6802187: *HIF1 alpha* (uniprotkb:Q16665) *physically interacts* (MI:0218) with *FIH1* (uniprotkb:Q9NWT6) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6802058: *HIF1 alpha* (uniprotkb:Q16665) *physically interacts* (MI:0218) with *HDAC4* (uniprotkb:P56524) by *pull down* (MI:0096)
MINT-6802021: *HIF1 alpha* (uniprotkb:Q61221) *physically interacts* (MI:0218) with *HDAC4* (uniprotkb:P56524) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6802036: *HIF1 alpha* (uniprotkb:Q61221) *physically interacts* (MI:0218) with *HDAC5* (uniprotkb:Q9UQL6) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6802102: *HIF1 alpha* (uniprotkb:Q16665) *physically interacts* (MI:0218) with *HDAC5* (uniprotkb:Q9UQL6) by *pull down* (MI:0096)
MINT-6802121, MINT-6802156: *P300* (uniprotkb:Q09472) *physically interacts* (MI:0218) with *HIF1 alpha* (uniprotkb:Q16665) by *anti bait coimmunoprecipitation* (MI:0006)

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1. Introduction

Hypoxia-inducible factor-1 (HIF-1) is a key transcriptional activator induced under hypoxic conditions that regulates cell adaptation and survival to hypoxia [1]. It is a heterodimer of HIF-1α and HIF-1β, which bind to the *cis*-acting hypoxia-response element (HREs) of the target promoters. HIF-1α is posttranslationally modified to regulate function of HIF-1 in response to the availability of oxygen [1,2]. Hydroxylation of Pro564 on the ODD by prolyl hydroxylase regulates stability of the HIF-1α protein [3]. HIF-1α is acetylated by the acetyltransferase, ARD1, and by p300/CBP-associated factor, although the role of the acetylation in HIF-1α function remains controversial [4,5]. Another pivotal modification could be hydroxylation of Asp803 in the C-terminal activating domain (C-TAD) by factor inhibiting HIF-1 (FIH-1), which blocks recruitment of transcriptional coactivators such as CBP/p300 under normoxic conditions, inhibiting the transactivation function of HIF-1 [6–8].

Acetylation of specific lysine residues on amino termini of the core histone, which is controlled by antagonistic activity of histone

Abbreviations: HIF-1, hypoxia-inducible factor-1; ODD, oxygen-dependent degradation; FIH-1, factor inhibiting HIF-1; HDAC, histone deacetylase; HRE, hypoxia-response element; C-TAD, C-terminal activating domain; ID, inhibitory domain; TSA, trichostatin A; pVHL, von Hippel–Lindau; RT-PCR, reverse transcriptase-polymerase chain reaction; GFP, green fluorescent protein; GST, glutathione S-transferase; si-RNA, small interfering-RNA; DFO, desferroxamine.

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doi:10.1016/j.febslet.2008.11.044

acetyltransferases and histone deacetylase (HDACs), is essential during activation of gene transcription [9]. The eukaryotic classical HDAC family is classified into three groups according to the phylogenetic and sequence similarity [9,10]. Class I HDAC family members such as HDAC1 and HDAC2 are closely related to the yeast transcriptional regulator, RPD3, while class II HDACs including HDAC4 and HDAC5 are closely related to the yeast deacetylase, HDA1. HDAC11 is classified into class IV, because of its low similarity to other classes [9]. Because HDACs are involved in tumorigenesis and angiogenesis, HDACs are among the most promising targets for treating various human cancers. Currently, the first-generation HDAC inhibitors are being tested in Phase I and II clinical trials [11,12].

Although HIF-1α is stabilized and VEGF is upregulated by overexpression of HDAC1 [13,14], the mechanism of cross-talk between HIF-1α and the HDAC is unclear. An HDAC inhibitor, trichostatin A (TSA), induced acetylation of HIF-1α in the oxygen-dependent degradation (ODD) domain [5], and it increased expression of p53 and von Hippel–Lindau (pVHL), which leads to degradation of HIF-1α [13]. HDAC inhibitors also induced degradation of HIF-1α by disrupting the HSP70/HSP90 axis function, which is a pVHL-independent mechanism [15]. Acetylation regulated HIF-1 function by targeting the HIF-1α/p300 complex, not HIF-1α directly [16]. Class II HDACs induce HIF-1α stability through a VHL-independent but proteasome-dependent pathway [17]. These controversies may represent the diverse roles of HDAC classes and subtypes in the regulation of HIF-1α. Here, we investigated the roles of HDAC4 and HDAC5 in the regulation of HIF-1α function and its associated mechanisms. HDAC4 and HDAC5 bound the inhibitory domain (ID) of HIF-1α and enhanced transactivation function through differential recruitment of p300 and FIH-1.

2. Materials and methods

2.1. Cells and cell culture

HepG2 (ATCC HB 8065), HeLa (ATCC CCL-2), NIH3T3 (ATCC CRL-1658), and HEK293 were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified eagle's medium or Iscove's modified Dulbecco's medium containing 10% fetal bovine serum at 37 °C in a 5% CO_2/95% air incubator. Cells were exposed to hypoxia (0.1% O_2) by incubating cells at 37 °C in 5% CO_2/10% H_2/85% N_2 anaerobic incubator (Forma Scientific, Waltham, MA). Hypoxia was also induced chemically by treating cells with 100 μM desferrioxamine (DFO).

2.2. Plasmids and transient transfection

The FLAG-tagged HDAC4, and HDAC5, the glutathione S-transferase (GST)-fused truncated HIF-1α, and green fluorescent protein (GFP-HIF-1α) were as previously described [18,19]. The reporter containing the Gal4 binding site, Gal4-*tk*-luc, and Gal4-HIF-1α, which contains the DNA-binding domain (1–147 amino acids) of yeast Gal4 linked to the full-length coding region of mouse HIF-1α, has been described [19]. Transient expression of proteins and reporter gene analyses were as previously described [14,19].

2.3. RT-PCR, Western blotting and immunoprecipitation

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described previously [14,19]. Western blotting and immunoprecipitation were performed as previously described using specific antibodies against HIF-1α, VEGF, HDAC4, HDAC5, FIH-1, β-actin (Santa Cruz Biotech), FLAG (Sigma–Aldrich), and α-tubulin (Calbiochem) [14,19].

2.4. Transfection of si-RNA duplexes

The small interfering-RNA (si-RNA) duplexes targeting HDAC4 (si-HDAC4; 5′-AAAUUACGGUCCAGGCUAATT-3′ and 5′-UUAGCCUGGACCGUAAUUUTT-3′), HDAC5 (si-HDAC5; 5′-GACUGUUAUUAGCACCUUUTT-3′ and 5′-AAAGGUGCUAAUAACAGUCTT-3′), and nonspecific si-RNA (si-control; 5′-GUUCAGCGUGUCCGGCGAGTT-3′ and 5′-CUCGCCGGACACGCUGAACTT-3′) were transfected as previously described [14].

2.5. Establishment of GFP-HIF-1α stable cell line

HeLa cells (1×10^4 cells per 60 cm^2 dish) were seeded and incubated overnight. After 48 h of transfection with 1 μg GFP-HIF-1α, G418 was added to a final concentration of 0.6 mg/ml. After 3 weeks of incubation in medium containing G418, selected colonies were transferred to 12-well plates. As cells grew, they were moved to a larger plate and maintained in G418-containing medium.

3. Results

3.1. HDAC4 and HDAC5 increase the transactivation function of HIF-1α

Based on the previous observation that inhibition of class II HDACs by valproic acid blocked the expression of HIF-1α [17], we examined how HDAC4 and HDAC5, which are class II HDACs, regulate activity of HIF-1α. When HDAC4 and HDAC5 were overexpressed in HeLa cells, protein and mRNA levels of HIF-1α were not

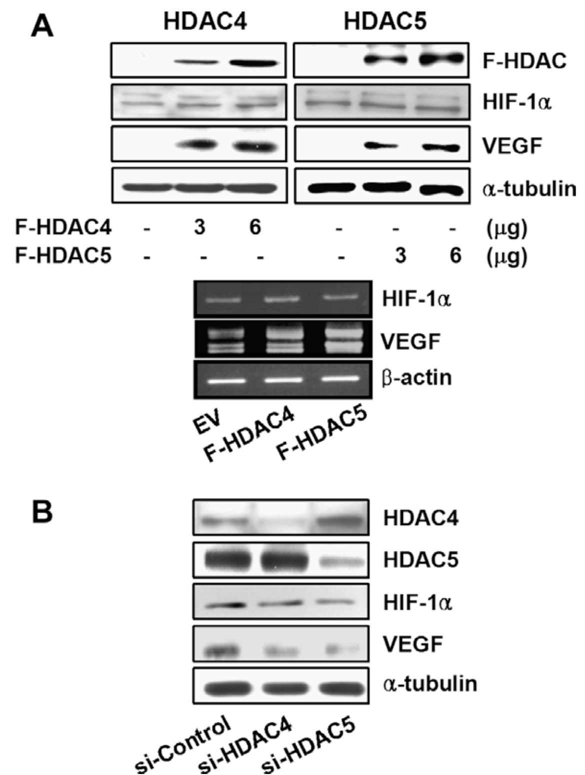

Fig. 1. Expression level of VEGF is regulated by HDAC4 and HDAC5. (A) HeLa cells were transfected with the indicated amount (upper) or 6 μg (lower) of pCMV-FLAG-HDAC or empty vector. After 24 h of transfection, whole cell lysates were prepared. The expression of proteins and transcripts were analyzed by western blot analysis (upper) and RT-PCR (lower), respectively. (B) HEK293 cells were transfected with 250 pmol of non-specific si-RNA (si-control), si-HDAC4 or si-HDAC5 for 48 h. The expression of proteins was analyzed by western blot analysis. One representative of at least three independent experiments with similar results is shown.

altered, whereas VEGF, an angiogenic target gene of HIF-1α, increased in a dose-dependent manner (Fig. 1A). When expression of HDAC4 and HDAC5 was blocked by RNA interference, expression of VEGF, but not HIF-1α, diminished (Fig. 1B). These results suggest that HDAC4 and HDAC5 increase expression of VEGF by enhancing transactivation by HIF-1α, instead of enhancing transcription or protein stability. Therefore, we tested whether HDAC4 and HDAC5 enhanced the transactivation function of HIF-1α using a Gal4-driven reporter system [19]. Cotransfection of the Gal4-HIF-1α plasmid with HDAC4 or HDAC5 significantly enhanced the Gal4-*tk*-luc reporter, similar to the activity induced by DFO, a hypoxia-mimicking agent, while HDAC1 did not (Fig. 2A). Treatment of either si-HDAC4 or si-HDAC5 significantly blocked the basal activity as well as the DFO-induced transcriptional activity of Gal4-HIF-1α (Fig. 2B), further demonstrating that HDAC4 and HDAC5 were required for transcriptional activation of HIF-1α.

3.2. HDAC4 and HDAC5 interact with HIF-1α through the ID

To investigate molecular mechanisms of the HDAC4- and HDAC5-induced transactivation by HIF-1α, we examined whether HDAC physically associated with HIF-1α. As shown in Fig. 3A, each HDAC4 and HDAC5 coimmunoprecipitated with HIF-1α. Next, we determined the interaction domains of HIF-1α using GST-fused truncated HIF-1α. The results showed that mainly the ID, but not the N-terminus (N), the ODD or the C-TAD, served as a binding site for both HDAC4 and HDAC5 (Fig. 3B; data not shown).

3.3. Differential bindings of HIF-1α to FIH-1 and P300 induces transactivation function of HIF-1α in the presence of HDAC4 and HDAC5

As FIH-1 binds to HIF-1α through ID and blocks binding of p300 on the C-TAD, which results in repression of HIF-1α function [8], we tested whether this binding was altered in the presence of HDACs. Coimmunoprecipitation data showed that binding of HIF-1α to FIH-1 decreased in the presence of HDAC4 or HDAC5. In contrast, binding of HIF-1α to p300 increased (Fig. 4A). These results indicate that HIF-1α dissociated from FIH-1 and that it recruits p300 in the presence of HDAC4 or HDAC5. When the expression of HDAC4 and HDAC5 was repressed by si-RNAs, the hypoxia-induced binding of HIF-1α to p300 was disappeared, whereas the binding of HIF-1α to FIH-1 was increased (Fig. 4B). Interestingly,

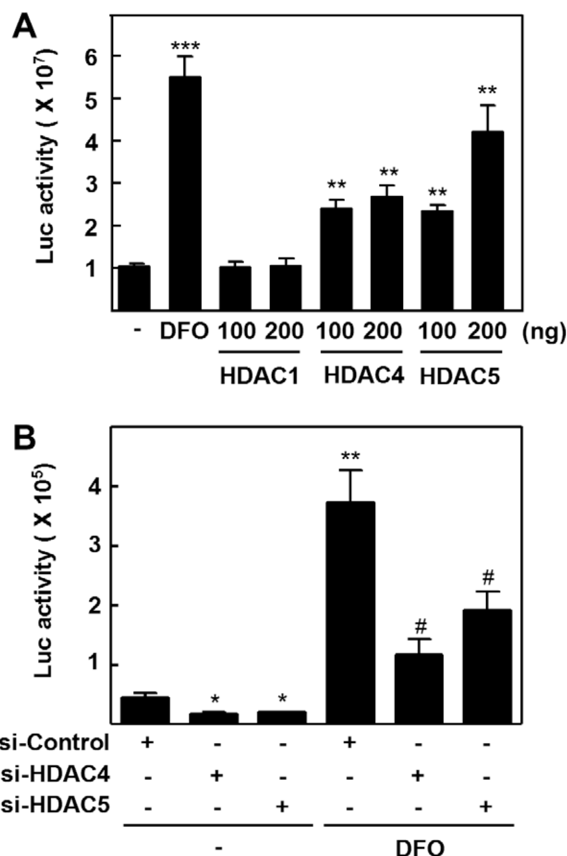

Fig. 2. HDAC4 and HDAC5 enhance transactivation function of HIF-1. (A) HepG2 cells were transfected with 150 ng Gal4-*tk*-luc plasmid, 50 ng Gal4-HIF-1α plasmid, and the indicated amount of pCMV-FLAG-HDAC. After 48 h of transfection, luciferase activity was measured and normalized for transfection efficiency using the corresponding β-galactosidase activity. Treatment with 100 μM DFO for 24 h was shown as positive control. Data shown are the mean ± S.E.M of three independent experiments ($^{**}P < 0.01$ and $^{***}P < 0.001$ vs. vehicle treated control). (B) HepG2 cells were cotransfected with 150 ng Gal4-*tk*-luc plasmid, 50 ng Gal4-HIF-1α plasmid and 20 pmole si-RNAs. After 24 h of transfection, cells were treated with 100 μM DFO for 24 h ($^{*}P < 0.05$ and $^{**}P < 0.01$ vs. si-control with vehicle; $^{\#}P < 0.05$ vs. si-control with DFO).

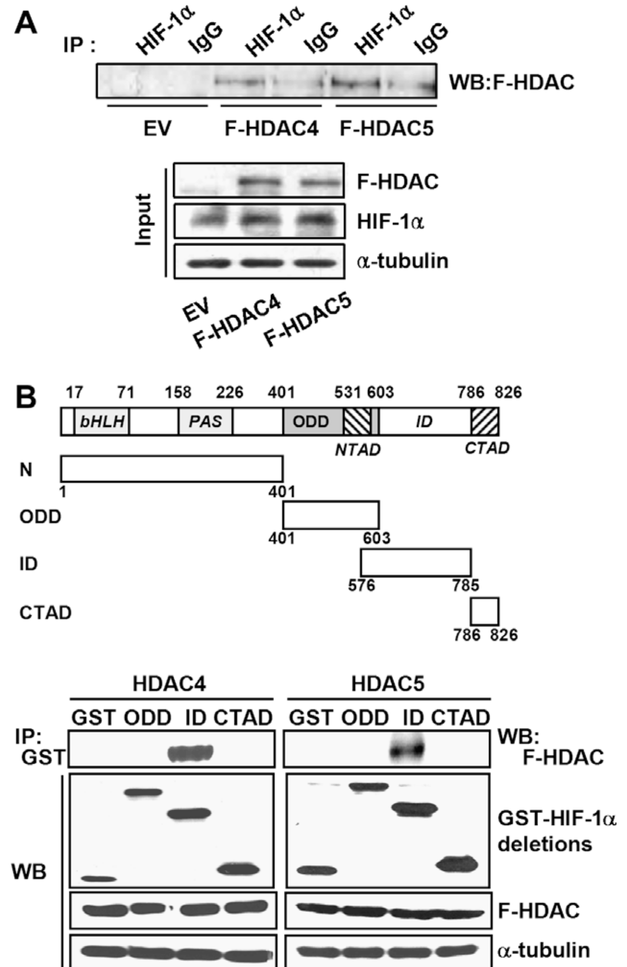

Fig. 3. Bindings of HIF-1α to p300 and to FIH-1 were changed in the presence of HDAC4 and HDAC5. (A) HDAC4 and HDAC5 interacted with HIF-1α. NIH3T3 cells were transfected with 9 μg pCMV-FLAG-HDAC or empty vector. 500 μg of whole cell lysates were immunoprecipitated (IP) with anti-HIF-1α antibody or normal IgG, and then probed using anti-FLAG antibody by western blot (WB) analysis. (B) Schematic representation of full-length and deletion HIF-1α constructs containing the basic helix–loop–helix (bHLH)/PER–ARNT–SIM (PAS), ODD, ID, N-terminal transactivation domain (NTAD), and CTAD (upper). NIH3T3 cells were transfected with 3 μg each pEBG-HIF-1α deletion construct and FLAG-HDAC or empty vector. Whole cell lysates were immunoprecipitated with anti-GST antibody, and then probed using anti-FLAG antibody. The expression of GST-HIF-1α deletions, FLAG-HDAC, and α-tubulin was analyzed by western blot analysis (lower).

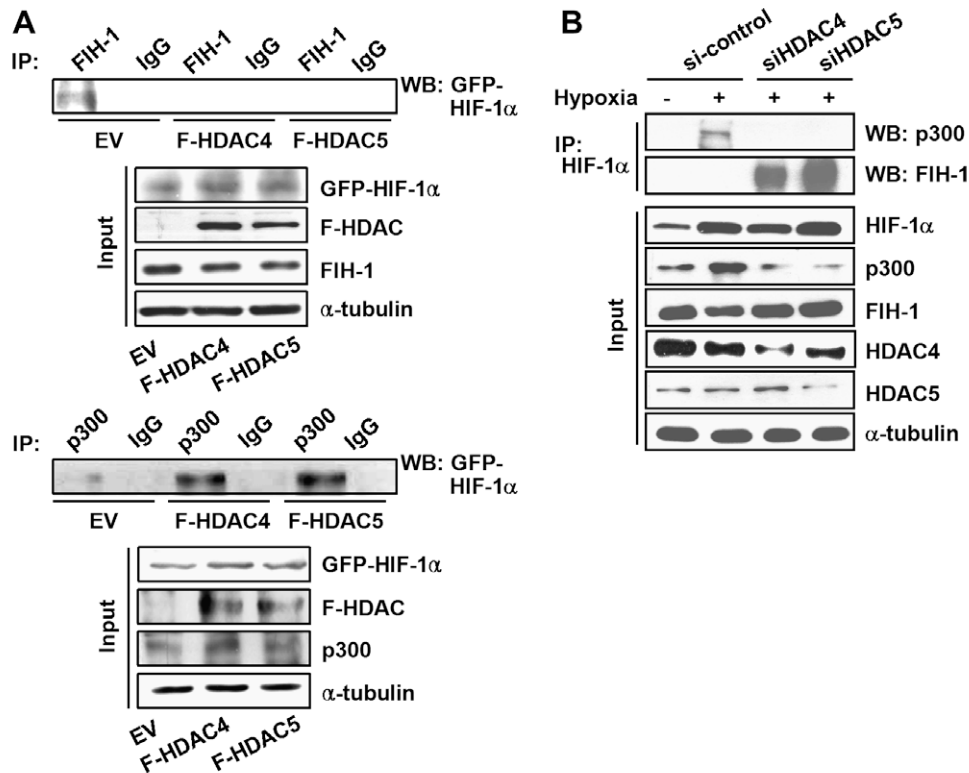

Fig. 4. Differential binding of HIF-1α to FIH-1 and p300 in the presence of HDAC4 and HDAC5. (A) GFP-HIF-1α stable cells were transfected with 6 μg pCMV-FLAG-HDAC or empty vector. Whole cell lysates were immunoprecipitated (IP) with anti-FIH-1 anti-p300, or normal IgG and then probed using anti-HIF-1α antibody. Expression of the indicated proteins was analyzed by western blotting as control. GFP did not interact with either FIH-1 or HIF-1α (data not shown). (B) HeLa cells were transfected with 50 nmol of si-control, si-HDAC4 or si-HDAC5 for 48 h, and then exposed to hypoxia. Whole cell lysates were immunoprecipitated (IP) with anti-HIF-1α and then probed using anti-p300 or anti-FIH antibody. Expression of the indicated proteins was analyzed by western blotting as control.

expression level of p300 was also decreased after si-HDAC4 and si-HDAC5 treatment, suggesting a potential regulation mechanism of p300 by the class II HDACs. Finally, the Gal4-*tk*-luc reporter activity that was induced by HDAC4 or HDAC5 was decreased by FIH-1 in a dose-dependent manner. In contrast, the reporter activity that was decreased by FIH-1 was recovered along with the expression of HDAC4 or HDAC5, dose dependently (Fig. 5). Together, these data demonstrated that HDAC4 and HDAC5 increased the transcriptional activity of HIF-1α by promoting dissociation of HIF-1α from FIH-1 and association with coactivator p300.

4. Discussion

HIF-1α and HDACs are over-expressed in various human cancers, and they are closely associated with malignant transformation and metastasis [1,12]. HDAC inhibitors possess anti-tumorigenic and anti-angiogenic effects, which may be mediated, at least in part, by the inhibiting function of HIF-1 and VEGF [13]. Thus far, HDACs have been demonstrated to regulate diverse aspects of HIF-1α function, such as protein stability, subcellular localization, and transactivation function [13–17,20,21], which suggest that each HDAC subtype may have a distinct mechanism for controlling HIF-1α. We demonstrated that HDAC4 and HDAC5-induced transactivation function of HIF-1α which involves differential recruitment of FIH-1 and p300.

Although the interplay between HDAC and HIF-1α has been well studied, it remains unclear [4,13–17]. Several distinct mechanisms have been described for the HDAC-induced activation of HIF-1. First, HDAC subtypes such as HDAC1 and HDAC3 enhanced HIF-1α protein stability through interaction with the ODD of HIF-1α [13,20]. In contrast, HDAC4 and HDAC6 induced HIF-1α protein stability *via* a pVHL-independent-, but proteasome-dependent pathway [17]. Second, HADC4, HDAC5 and HDAC7 increased transactivation function of HIF-1 by recruiting p300 (Figs. 4 and 5) [21]. Third, HDAC7 increased transcriptional activity of HIF-1α by promoting nuclear translocation under hypoxic conditions [21]. In addition, indirect mechanisms were proposed in that the HDAC inhibitor suppressed transactivation function of HIF-1α by hyperacetylation of p300, not HIF-1α, which blocked formation of the HIF-1α-p300 complex [16]. Inhibition of HDAC6 also induced degradation of HIF-1α *via* hyperacetylation of Hsp90 which induced binding of Hsp70 to HIF-1α [15]. Together, HDACs employ multiple strategies for modulation of HIF-1α to ensure HIF-1α activity is maintained under hypoxia. A variety of other stimuli that activate HIF-1α, such as growth factors, pH, and mechanical stresses, may also use combinations of HDAC subtypes to achieve a specific regulation of HIF-1α in a stimulus-specific manner.

FIH-1 is originally known as a member of the superfamily 2-oxoglutarate and Fe(II)-dependent dioxygenase [22]. FIH-1 has emerged as a critical oxygen sensor in the hypoxic response pathway because it hydroxylates Asp803 on the C-TAD of HIF-1α in an oxygen-dependent manner [7]. Consequently, it blocks recruitment of transcriptional coactivator proteins such as CBP/p300 under normoxic conditions, which results in inhibition of transactivation function of HIF-1 [8]. Here, HDAC4 and HDAC5 induce transactivation function of HIF-1 by inhibiting association of FIH-1 to HIF-1, further confirming the inhibitory function of FIH-1 in hypoxia signaling (Fig. 3). Bortezomib, a proteosome inhibitor, may utilize a similar mechanism in that it represses HIF-1α through FIH-1-mediated inhibition of p300 recruitment [23]. Recently, it was shown that FIH-1 was a substrate for Siah-1, a member of the E3 ubiqutin ligase family, and was subjected to

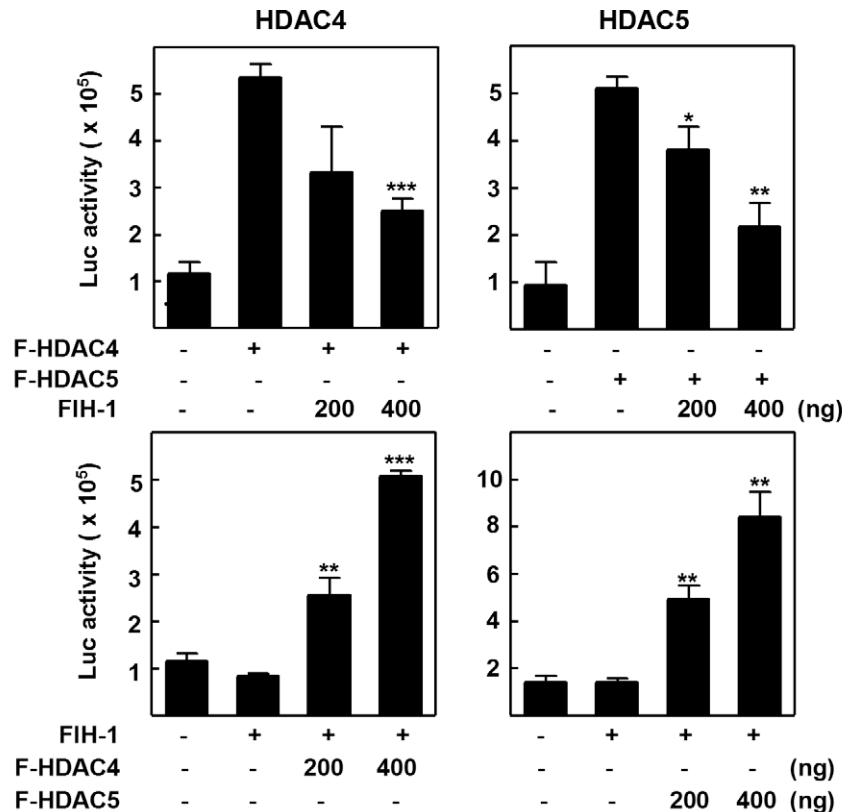

Fig. 5. HDAC4 and HDAC5 recover the transcriptional activity of HIF-1α which is repressed by FIH-1. HepG2 cells were co-transfected with 150 ng Gal4-tk-luc and 50 ng Gal4-HIF-1α with the indicated combinations of expression vectors for FIH-1 and HDAC4. After 48 h of transfection, luciferase activity was measured and normalized for transfection efficiency using the corresponding β-galactosidase activity. Data shown are the mean ± SEM of three independent determinations ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

ubiquitination/proteasomal degradation [24]. It may be interesting to test whether HDACs are also involved in deacetylation of FIH-1, which may induce the Siah-mediated degradation of FIH-1. FIH-1 has also been implicated in inflammation, because it hydroxylates asparaginyl residues within the IκB protein, implying that hydroxylation of intercellular proteins by FIH-1 is broader than previously thought [25]. The potential involvement of HDAC4 and HDAC5 in the new aspect of FIH-1 biology needs to be further investigated.

Currently, efforts are being made to understand the function of HDACs and to develop potent and subtype-specific HDAC inhibitors. Importantly, differential expression of HDAC4 and HDAC5 was noticed in breast tumors and colorectal tumors [26], thus studies on the biological function of HDAC subtypes related to HIF-1α may affect strategies for hypoxia-associated human diseases.

Acknowledgments

We are grateful to Dr. Tso-Pang Yao for the expression vectors for HDAC4 and HDAC5. This study was supported by grants from the KOSEF (2006-02634), and the SRC/ERC (R11-2007-107-01001-0).

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