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Dimethyl fumarate inhibits the expression and function of hypoxia-inducible factor-1α (HIF-1α)

Guangzong Zhao [a,b], Yao Liu [c], Jun Fang [b], Yunzhen Chen [a,*], Huazhuang Li [b], Kehai Gao [b]

[a] Department of Orthopaedics, Qilu Hospital of Shandong University, China
[b] Department of Orthopaedics, Yidu Central Hospital of Weifang, China
[c] Department of Obstetrics and Gynecology, Yidu Central Hospital of Weifang, China

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ABSTRACT

Osteocyte hypoxia has been induced by skeletal unloading and fracture. Hypoxia-dependent regulation of gene expression is mediated by hypoxia-sensitive transcription factors such as hypoxia-inducible factor-1α (HIF-1α). Dimethyl fumarate (DMF) is a recently approved first-line therapy for multiple sclerosis. However, the role of DMF in regulating HIF-1α expression and function has not been evaluated. In this study, we found that DMF inhibited hypoxia-induced expression of HIF-1α and its target genes such as interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF) in MC3T3 E1 cells. Mechanistically, DMF promoted HIF-1α degradation in a proteasome-dependent but von Hippel–Lindau (VHL) protein-independent manner. Importantly, we found that DMF disrupted the interaction between HIF-1α and its chaperone heat shock protein 90 (Hsp90) but promoted the interaction between HIF-1α and the receptor of activated protein kinase C (RACK1). These data suggest that DMF might promote degradation of HIF-1α by affecting its folding and maturation. Based on these observations, we conclude that DMF is a novel inhibitor of HIF-1α.

1. Introduction

Reduced O_2 tension has been shown to have significant effects on bone cell physiology. It has been shown that skeletal unloading is able to induce osteocyte hypoxia [1]. Moreover, a fracture may cause a significant decrease in O_2 tension in bone cells due to disruption of the vasculature [2]. In bone cells, hypoxia has been shown to up-regulate the expression of several important genes, including vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8), which facilitate new blood vessel formation and mediate inflammation response [3]. Multiple lines of evidence have shown that hypoxia-dependent regulation of gene expression takes place at the transcriptional level and is mediated by hypoxia-sensitive transcription factors such as hypoxia-inducible factor-1α (HIF-1α). HIF-1α protein levels are tightly regulated by oxygen tension via controlled proteolysis [4]. On one hand, recognition of hydroxylated HIF-1α by the von Hippel–Lindau tumor suppressor (VHL) protein recruits an E3 ubiquitin ligase complex targeting HIF-1α for proteasomal degradation [5]. On the other hand, heat shock protein 90 (Hsp90), a molecular chaperone that protects client proteins from misfolding and degradation, is able to regulate HIF-1α degradation. Hsp90 binds to the HIF-1α PAS domain. Notably, the Hsp90 inhibitors geldanamycin and 17-allylaminogeldanamycin induce proteasomal degradation of HIF-1α even in VHL-deficient cells [6]. In addition, the receptor of activated protein kinase C (RACK1), a HIF-1α-interacting protein, has been recently shown to promote VHL-independent proteasomal degradation of HIF-1α by competing with Hsp90 for binding to HIF-1α [7].

Dimethyl fumarate (DMF), a recently approved first-line therapy for multiple sclerosis, is an oral tablet thought to act by decreasing the expression of NF-κB dependent genes [8]. Treatment with DMF has been shown to reduce the expression of proinflammatory cytokines such as TNFα, IL-1β, and IL-6 in glial cells [9]. In addition, DMF has been shown to mediate a strong antioxidant effect via the Nrf2 signaling pathway. There is increased expression of important enzymes with antioxidant effects such as NQO1, HO-1, and GCLC [10]. Both proinflammatory response and oxidative stress take place in the process of hypoxia. However, little information regarding the physiological roles of DMF in hypoxia, especially in regulating the expression and function of HIF-1α has been reported in previous literatures. In this study, we demonstrate that DMF is a novel inhibitor of HIF-1α expression/function.

* Corresponding author. Address: Department of Orthopaedics, Qilu Hospital of Shandong University, No. 107, Wen Hua Xi Road, Ji'nan, Shandong province 250012, China. Fax: +86 531 82169114.
E-mail address: yzchen6501@hotmail.com (Y. Chen).

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2. Materials and methods

2.1. Cell culture

Mouse osteoblastic cells MC3T3-E1 (clone 14) were maintained in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S). Cells were pretreated with DMF at various concentrations from 10 to 100 μM for 24 h before hypoxia in the presence or absence of specific proteasome inhibitors MG132 or N-acetyl-leucyl-leucyl-norleucinal. Then media was replaced with α-MEM containing 0.1% FBS and 1% P/S, pre-conditioned in 1% or 21% O_2 tension for various time from 1 to 24 h. For ambient (21%) O_2 tension experiments, cells were cultured in a standard humidified incubator at 37 °C with a 95% air and 5% CO_2 atmosphere. For hypoxia experiments, cells were cultured in humidified incubators at 37 °C with 5% CO_2 and with O_2 tension reduced to 1% using supplemental N_2.

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from MC3T3 E1 cells was isolated using Trizol reagent (Invitrogen) according to the manual instructions. 2 μg total RNA was used to synthesize cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA). Synthesized cDNA by reverse transcriptase polymerase chain reaction (RT-PCR) was used to study the expression of VEGF and IL-8 in MC3 T3 E1 cells. The primers used for VEGF are: forward (5′-CTAT GTGCTGGCTTTGGTGA-3′) and reverse (5′-GCCGGCGCCCTCCAT-3′). The primers used for IL-8 are: forward (5′-CTGGCCGTGGCTCT CTTG-3′) and reverse (5′-CCTTGGCAAAACTGCACCTT-3′). The primers used for HIF-1α are: forward (5′-GTCGGACAGCCTCACCAAAC AG-3′) and reverse (5′-TAGGTAGTGAGCCACCAGTGTCC-3′). The primers used for β-actin are: forward (5′-CTGTCGAGTCGCGTCCA CCC-3′), and reverse (5′-GCTTTGCACATGCCGGAGCC-3′).

2.3. Immunoprecipitation and Western blot assays

For immunoprecipitation assays, cells were lysed by cell lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, protease inhibitor mixture, sodium orthovanadate, and sodium fluoride. Protein concentration was determined by Pierce BCA protein assay kit (Pierce, USA). 1 mg whole cell lysates to form the antigen–antibody complex was used to incubate with 5 μg of anti-AIP1 or 14-3-3 antibody (Cell signaling, USA) overnight at 4 °C. Protein A/G-agarose beads (Santa Cruz) were used to precipitate the formed antigen–antibody complex for 2 h at 4 °C. Then the beads were eluted in SDS sample buffer. Protein samples were subjected to 10% SDS–PAGE and electrotransferred onto and subjected to SDS–PAGE. For immunoblot assays, the cells were lysed in cell lysis buffer (Cell signaling, USA). Then cellular lysates were subjected to SDS–PAGE followed by immunoblot assays as described previously [11].

2.4. Enzyme-linked immunosorbent assay (ELISA)

Mouse VEGF and IL-8ELISA kits were purchased from R & D Systems. Briefly, MC3T3 E1 cells were cultured in 6-well plates and exposed to hypoxia for indicated times. The supernatants were harvested and assayed for VEGF or IL-8 by ELISA according to the manufacturer's instructions.

2.5. HIF-1α pulse labeling

The cells were washed and maintained with cysteine/methionine-free α-minimum essential medium (α-MEM) for 2 h. Then cells were pulse labeled with [35S] methionine–cysteine (88 mCi/ml) and collected after 15 and 45 min. For the hypoxic cells, the washing and incubation media were preequilibrated in hypoxia, and all manipulations were carried out in the hypoxia chamber. Total protein lysates were obtained and immunoprecipitated by HIF-1α antibody. After eluted from the beads, radiolabeled HIF-1α protein was subjected to SDS–PAGE. Gels were dried using the HydroTech gel drying apparatus (Bio-Rad Laboratories, USA) and autoradiographed. This experiment was repeated for 4 times.

2.6. Statistical analysis

All of the results are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's HSD test. Differences between means were considered statistically significant at $P < 0.05$.

3. Results

DMF is the methyl ester of fumaric acid. The molecular structure of DMF is shown in Fig. 1A. We first performed a time course study and assessed the expression of HIF-1by Western blot analysis in MC3T3 E1 cells exposed to hypoxia (1% O_2) for 1, 4, 8, and 24 h. As shown in Fig. 1B, HIF-1α protein expression was induced at 1 h and peaked 8 h after hypoxic stimulation. Therefore, we chose 8 h of hypoxia for the following experiments. Then we explored whether DMF had a role in regulating HIF-1α. Our results indicated that pretreatment with DMF significantly blunted the hypoxia-induced accumulation of HIF-1α protein (Fig. 1C).

We then investigated whether DMF has a role in the alternations of HIF-1α's target genes. IL-8 and VEGF have been considered as HIF-1α's target genes in the process of hypoxia. The cells and culture media were harvested and subjected to RT-PCR analysis and ELISA, respectively. As shown in Fig. 2A, administration of DMF inhibits both basal and hypoxia-induced IL-8 mRNA expression. Consistently, ELISA revealed that DMF inhibited IL-8 protein secretion from MC3T3 E1 cells (Fig. 2B). In addition, pretreatment with DMF has also been found to inhibit the hypoxia-mediated VEGF mRNA expression (Fig. 2C) and protein secretion (Fig. 2D).

To further understand the mechanisms by which DMF decreases HIF-1α protein levels, we examined the role of the proteasome system in DMF-induced HIF-1α degradation by use of pharmacologic proteasome inhibitors. Fig. 3A shows that MG132, a specific cell-permeable proteasome inhibitor, rescued HIF-1α degradation by DMF. In addition, similar rescue was observed using the highly specific proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal. VHL is the substrate recognition component of an E3 ubiquitin ligase and functions as a master regulator of HIF activity by targeting the hydroxylated HIF-α subunit for ubiquitylation and rapid proteasomal degradation under normoxic conditions [12]. We next investigated whether DMF can promote HIF-1α protein degradation in the absence of VHL in MC3T3 E1 cells. Successful knockdown of VHL is shown in Fig. 3B. As expected, silence of VHL resulted in an induction of HIF-1α protein under normoxic conditions. However, DMF inhibited HIF-1α protein expression in the absence of VHL under normoxic and hypoxic conditions (Fig. 3C). These data indicate that the effect of DMF on HIF-1α degradation is independent on VHL.

The half-life of HIF-1α is also regulated by the competitive binding of either heat shock protein 90 (HSP90), which stabilizes the protein, or the anchoring protein (RACK1), which interacts with Elongin C, thereby promoting HIF-1α ubiquitination and degradation that is independent of PHD2 and VHL [13]. Then we investigated the effect of DMF on the interaction between HIF-1α and Hsp90. MC3T3 E1 cells were exposed to hypoxia for 8 h with treatment of MG132 to reverse HIF-1α protein degradation by DMF.

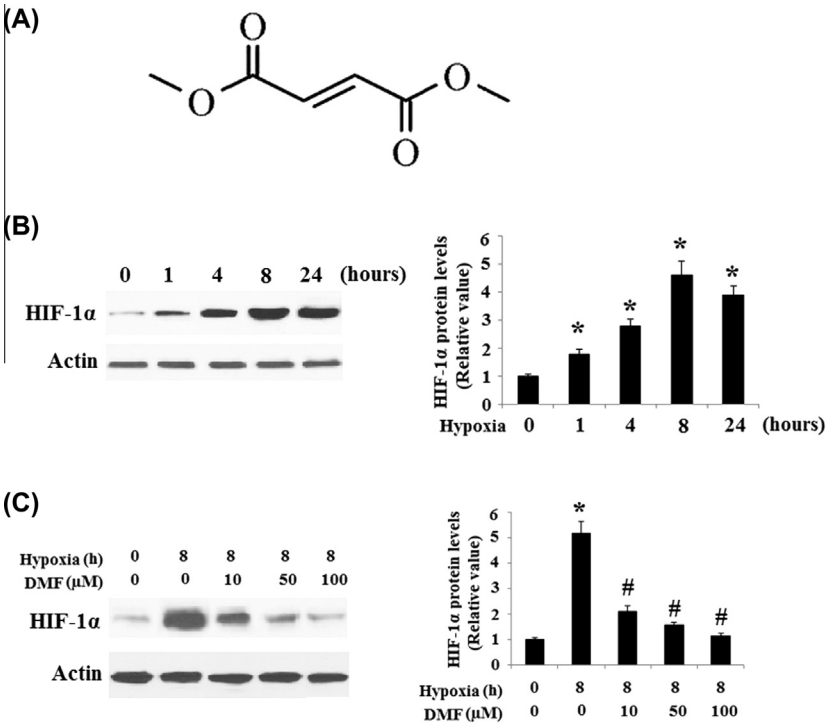

Fig. 1. Effect of dimethyl fumarate (DMF) on HIF-1α protein expression. (A) Molecular structure of DMF. (B) MC3T3 E1cells were exposed to hypoxia (1% O_2) for the indicated time. The cells were harvested and followed by Western blot analysis. A representative blot is shown of HIF-1α (*, $P < 0.01$ vs. Normoxia, $n = 4$). (C) Pretreatment with DMF inhibits HIF-1α expression in response to hypoxia in a dose dependent manner. MC3T3 E1 cells were pretreated with DMF at various concentrations for 24 h and were exposed to normoxia or hypoxia (1% O_2) for 8 h. The cell lysates were harvested and subjected to Western blot analysis (*, $P < 0.01$ vs. Normoxia; #, $P < 0.01$ vs. Hypoxia, $n = 4$).

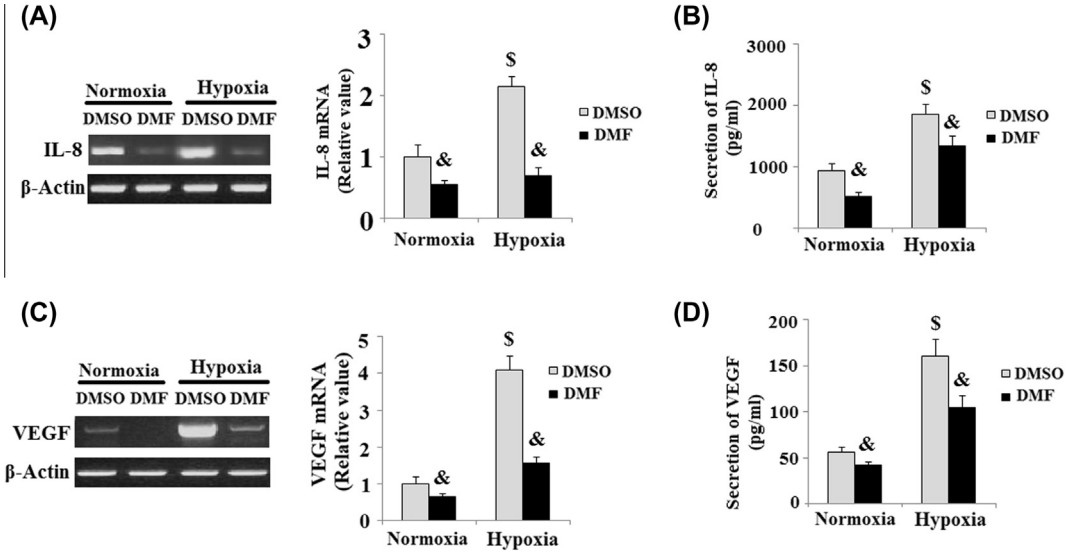

Fig. 2. Dimethyl fumarate (DMF) regulates the induction of hypoxia-mediated gene IL-8 and VEGF. MC3T3 E1 cells were pretreated with DMF (50 μM) for 24 h and were exposed to normoxia or hypoxia (1% O_2) for 8 h. (A) mRNA levels of IL-8 was determined by RT-PCR. (B) Secretion of IL-8 was determined by Elisa assay. (C) mRNA levels of VEGF was determined by RT-PCR. (D) Secretion of VEGF was determined by an Elisa assay ($^{\&}$, $P < 0.01$vs. data within the group; $^{\$}$, $P < 0.01$vs. data in the different group, $n = 3$–5).

Immunoprecipitation with anti-HIF-1α antibody and Western blot with an antibody against Hsp90 showed that DMF inhibited the ability of HIF-1α to form a complex with Hsp90 (Fig. 3D). In contrast, immunoprecipitation with anti-HIF-1α antibody and Western blot with an antibody against RACK1 showed that DMF promotes the interaction between HIF-1α and RACK1 (Fig. 3E). These findings suggest that DMF promotes degradation of HIF-1α by disrupting its ability to interact with the Hsp90 chaperone complex but promoting its interaction with RACK1, thereby affecting its folding and maturation.

To determine whether the reduction of HIF-1α by DMF occurs at the transcriptional level, we analyzed HIF-1α mRNA levels in DMF treated cells. In normoxia, RT-PCR indicated that HIF-1α mRNA expression was not affected by treatment with DMF at different concentrations (Fig. 4A). To investigate whether the hypoxic regulation of HIF-1α mRNA is affected by DMF, cells were cultured in

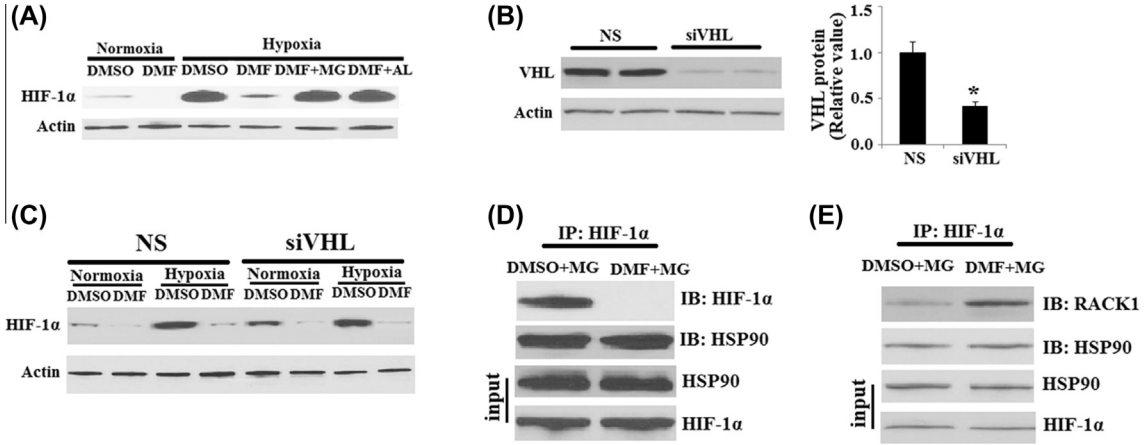

Fig. 3. HIF-1α degradation by DMF is VHL-independent but mediated by the proteasome system. DMF disrupts the interaction between HIF-1α and Hsp90 but promotes the interaction between HIF-1α and RACK1. (A) Proteasome inhibitors rescue dimethyl fumarate (DMF)-mediated HIF-1α degradation. MC3T3 E1 cells pretreated with DMF (50 μM) were exposed to normoxia or hypoxia (1% O_2) for 8 h in the absence or presence of proteasome inhibitors (MG, MG132 5 μM; AL, N-acetyl-leucyl-leucyl-norleucinal 30 μM). Representative blots of four independent experiments are shown. (B) MC3T3 E1 cells were transfected with non-specific RNA (NS) or VHL siRNA (siVHL). Western blot analysis revealed successful knockdown of VHL (*, $p < 0.01$ vs. NS group, $n = 4$). (C) DMF inhibits HIF-1α protein expression in a VHL-independent fashion. Representative blots of four independent experiments are shown. (D) MC3T3 E1 cells were stimulated by hypoxia (1% O_2) for 8 h in the presence of MG132 (5 μM). The cell lysates were immunoprecipitated (IP) by anti-HIF-1α antibody. The precipitate was immunoblotted (IB) with anti-HIF-1α and anti-Hsp90 antibodies. (E) The precipitate was immunoblotted (IB) with anti-HIF-1α and anti-RACK1 antibodies. Representative blots of four independent experiments are shown.

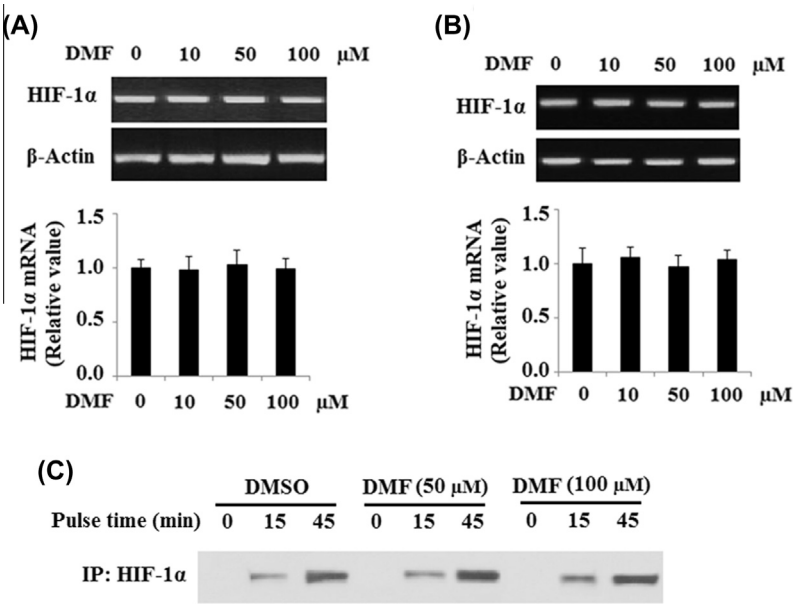

Fig. 4. Dimethyl fumarate (DMF) didn't change the intracellular HIF-1α transcription and translation. (A) Regular-PCR analysis of HIF-1α mRNA levels in MC3T3 E1 cells in normoxia in the presence or absence of DMF. (B) Regular-PCR analysis of HIF-1α mRNA levels in MC3T3 E1 cells exposed to hypoxia for 8 h in the presence or absence of DMF. (C) HIF-1α protein synthesis rate was determined in cells exposed to [35S]-labeled methionine and cysteine for the indicated time. Pulse analysis results revealed that DMF didn't reduce the HIF-1α protein synthesis rate. Experiments were repeated for 4 times.

hypoxia for 8 h. However, administration of DMF didn't lead to a significant change in mRNA levels of HIF-1α (Fig. 4B). These data suggest that DMF does not regulate HIF-1α expression at transcriptional levels in either normoxia or hypoxia.

HIF-1α is a short half-life protein. Therefore, the protein expression level of HIF-1α in cells results from a balance between protein synthesis by translation and degradation. To investigate whether DMF reduced HIF-1α protein expression by affecting its translational rate, we determined the effect of DMF on HIF-1α translation by comparing the rate of HIF-1α synthesis in DMF-treated or untreated cells using [35S]-labeled methionine and cysteine in pulse analysis. However, as shown in Fig. 4C, HIF-1α protein synthesis rate was almost identical in control cells and DMF (50 or 100 μM) treated cells, indicating that DMF treatment didn't affect HIF-1α protein synthesis.

4. Discussion

Altered oxygen tension in the environment surrounding bone cells has been implicated in conditions of development, disuse, and skeletal fracture [1]. Indeed, the oxygen tension at a fracture site has been reported to be as low as 0.8% [14]. Hypoxia promotes adaptive changes in gene expression, primarily through stabilization of the HIF-α transcription factors. The HIF-1 transcription

factor consists of an O_2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit. HIF-1α is able to be hydroxylated in two proline residues. This process is catalyzed by proline hydroxylase in the presence of O_2 and Fe^{2+}. Recognition of hydroxylated HIF-1α by the VHL recruits an E3 ubiquitin ligase complex targeting HIF-1α for proteasomal degradation [15]. Interestingly, DMF promoted HIF-1α degradation in a VHL-independent manner. In addition, HIF-1α required HSP90 for stability, folding and trafficking. The Hsp90 inhibitors geldanamycin and 17-allylaminogeldanamycin induce proteasomal degradation of HIF-1α even in VHL-deficient cells [16]. Notably, RACK1 is able to promote VHL-independent proteasomal degradation of HIF-1α by competing with Hsp90 for binding to HIF-1α [7]. Importantly, our results indicated that DMF disrupts the interaction of HIF-1α with the Hsp90 chaperone complex but promotes its interaction with RACK1. DMF may affect effectors of both Hsp90 and RACK1 function. Further studies will provide a complete picture to elucidate the mechanism underlying this observation.

In reduced oxygen conditions, HIF-1 binds to hypoxia-responsive elements on target promoters to activate gene expression. In addition to the HIF-1-dependent mechanisms, hypoxia-mediated genes expression is also known to be activated through an HIF-1-independent Mechanism. For example, induction of IL-8 might be dependent on the NF-κB pathway as well as the HIF-1-dependent pathway under hypoxic conditions [17]. Consistent with the findings in the current study, previous studies have demonstrated that DMF exerts NF-κB function through inhibiting co-activator recruitment [18]. Both hypoxia-inducible genes IL-8 and VEGF are involved in the process of angiogenesis. The inhibitory effects of DMF on IL-8 and VEGF suggest a potential role of DMF in regulating hypoxia-mediated angiogenesis. Indeed, DMF has been shown to suppress angiogenesis through inhibiting the expression of the vascular endothelial growth factor receptor-2 (VEGFR2) [19].

In summary, we demonstrate here for the first time that DMF is a novel inhibitor of HIF-1α by changing its functional interaction with chaperone protein HSP90 and RACK1. Our study provides a further insight into the pharmacological function of DMF in the process of hypoxia.

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