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Dibenzoylmethane, a natural dietary compound, induces HIF-1α and increases expression of VEGF

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is the major transcription factor activated during hypoxia. It is composed of HIF-1α and HIF-1β subunits. While HIF-1β is constitutively expressed, HIF-1α is targeted to proteasome degradation under normoxic conditions. Under hypoxia, HIF-1α is stabilized and heterodimerizes with HIF-1β. Iron chelators have also been reported to stabilize HIF-1α protein and activate HIF-1. In this study, we investigated the effects of dibenzoylmethane (DBM), a natural dietary compound and an iron chelator, on HIF-1 pathway. We found that DBM increases HIF-1α protein levels in a dose- and time-dependent manner. This induction was accompanied with activation of HIF-1, measured by reporter gene assay and increased production of its downstream target, the vascular endothelial growth factor. Mechanistically, HIF-1α was stabilized by DBM at a step prior to ubiquitination. The effect of DBM on HIF-1 and its low toxicity profile might be therapeutically beneficial in ischemic diseases.

Keywords: Dibenzoylmethane; VEGF; HIF-1α; Prostate cancer cells; Cardiomyocyte

The cellular response to hypoxia is controlled to a large degree by the transcription factor HIF-1, consisting of two subunits, HIF-1α and HIF-1β (ARNT, arylhydrocarbon receptor nuclear translocator). Whereas the HIF-1β is constitutively expressed, HIF-1α is constitutively expressed and degraded through the proteasome pathway [1]. HIF-1α activity is under the regulation of signaling pathways, both via the phosphatidylinositol-3-kinase (PI3K) signaling [2–6] and the mitogen-activated protein kinase (MAPK) pathways [7,8]. Among the growth factors that can stimulate expression in some cells is angiotensin II that induces levels surpassing those of hypoxia [9]. With angiotensin II there was an increase in the rate of HIF-1α mRNA transcription via the protein kinase C pathway and in addition increase in the rate of translation by activating reactive oxygen species and the PI3K pathway [9]. Under normoxic conditions HIF-1α has a brief half-life and is rapidly degraded. This occurs by a series of steps. HIF-1α protein is hydroxylated on proline residues 402 and 564 [10–12] by a specific HIF-prolyl hydroxylases. The proline hydroxylase activity requires iron and oxygen [13,14]. The hydroxylated protein is recognized by the von Hippel-Lindau tumor suppressor protein (pVHL), which functions as E3 ubiquitin ligase. This interaction between HIF-1α and pVHL is further accelerated by acetylation of lysine residue 532 through an *N*-acetyltransferase, ARD1 [15]. Once the hydroxylated HIF-1α is ubiquinated it is rapidly degraded by the proteasome.

Under hypoxia the proline hydroxylase is not active and HIF-1α is not hydroxylated preventing the interaction with pVHL and ubiquitination. In the absence of degradation HIF-1α levels increase and HIF-1α is then translocated [16] to the nucleus where it heterodimerizes with HIF-1β. The activated HIF-1 drives transcription a battery of more than 40 genes whose products play a pivotal role in adaptation and survival under hypoxia [17–21].

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Low molecular weight compounds have been described that inhibit HIF-1α prolyl hydroxylase activity in vitro leading to increased levels of HIF-1α and the production of its downstream target including VEGF [22]. In addition, cobalt chloride ($CoCl_2$) and iron chelators such as desferroximine (DFO) have been shown to increase HIF-1α levels by preventing degradation [23], but have had limited practical utility due to toxicity. Recently, a mycobacterial iron chelator, desferrioxochelin, was found to induce HIF-1 and -2, NIP3, and VEGF in cancer cell lines [24]. In this study, we have tested the effects of a dietary component [25] dibenzoylmethane (DBM), an iron chelator [26], on HIF-1 activity in cell culture systems. Our experiments have shown that DBM is able to induce HIF-1α protein levels by preventing its degradation and dramatically increases HIF-1 transcriptional activation as well as inducing the secretion of VEGF under normoxic conditions. The capacity to induce VEGF under normoxic conditions, rather than responding to hypoxia, may have potential use in situations requiring re-vascularization.

Materials and methods

Tissue culture and reagents. Human prostate cancer cell lines LNCaP and PC-3 were maintained in RPMI 1640 culture medium and HEK 293 cells were maintained in DMEM culture medium. All media were supplemented with 10% fetal calf serum and cells were cultured at 37 °C in a humidified atmosphere and 5% CO_2 in air. Neonatal rat cardiomyocyte primary cultures were performed as described [27]. Briefly, hearts from 1- to 3-day-old Wistar rats were minced and dissociated with 0.08% trypsin in phosphate buffered saline (PBS). The cells were incubated in 75 cm^2 culture dishes at 37 °C for 1.5 h. The non-attached viable cells were collected and seeded onto a new dish in DMEM culture medium. After 6 h, 10 μM cytosine arabinoside was added for 24 h to inhibit nonmyocyte growth. For hypoxic exposure, cells were placed in a sealed modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with 1% O_2, 5% CO_2, and 94% N_2 (1% O_2).

DBM, dibenzoylpropane (DBP), curcumin, and $CoCl_2$ were obtained from Sigma–Aldrich (St. Louis, MO). Cycloheximide (CHX) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Mouse monoclonal anti-HIF-1α antibody was obtained from BD Transduction Laboratories (Lexington, KY). Monoclonal antibody against HIF-1β was obtained from Novus Biological (Littleton, CO). Rabbit polyclonal antibodies against ubiquitin and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal human antibody to human topoisomerase I (TOPO-I) was obtained from TopoGen (Columbus, OH). Secondary antibodies were horseradish peroxidase-conjugated and purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

DBM treatment. Cells were grown in six-well culture plates until 70% confluent. The media were replaced with fresh media containing vehicle (DMSO, dimethyl sulfoxide) or DBM (0.1 M stock solutions in DMSO). After 4 h at 37 °C the cells were collected and whole cell extracts (WCEs) or nuclear extracts (NEs) were prepared [28].

Protein isolation and Western blot analysis. Cells were washed twice with PBS at 4 °C and centrifuged at 500*g* for 5 min. NEs were prepared by resuspension in 10 mM Tris–HCl, pH 7.5, 1.5 mM $MgCl_2$, and 10 mM KCl with 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotonin, 2 μg/ml pepstatin, and 1 mM Na_3VO_4. The cells were kept on ice for 10 min. The nuclei were collected by centrifugation at 17,000*g* for 10 min at 4 °C. WCEs were prepared by lysing the cells with 100 mM potassium phosphate, pH 7.8, and 0.2% Triton X-100 supplemented with the above protease and phosphatase inhibitors. Samples from the WCEs or NEs were subjected to protein electrophoresis (30–60 μg of protein) with 7.5% SDS–PAGE and transferred to nitrocellulose membranes (BioRad, Richmond CA) by electrotransfer. The Western blots were developed with primary antibody and visualized by secondary antibody and enhanced chemoluminescence reagent (Amersham Biosciences, Piscataway, NJ). Membranes were treated with Western blot stripping buffer (Pierce, Rockford, IL) and re-probed with TOPO-I for NE and actin for WCE antibody to determine the equivalence of loading.

ELISA for VEGF. Culture media were collected, centrifuged to remove cellular debris, and stored at −80 °C until assayed for VEGF. VEGF assay was performed using a commercially available ELISA Kit (R&D Systems, Minneapolis, MN). Results between wells were standardized according to the amount of VEGF per total protein/well as measured in cell lysates or per cell number in each well and expressed as picogram of VEGF protein/ml supernatant per milligram protein or per 10^5 cells, respectively.

Transient transfections and reporter gene assay. HEK 293 cells were grown in six-well plates and transfected in triplicate with 1 μg/well of pBI-GL V6L containing hypoxia response element derived from the promoter of *VEGF* gene using the GenePorter transfection (Gene Therapy Systems, San Diego, CA) as described [29,30]. After 5 h the media were replaced and the cells were incubated overnight. The cells were washed twice in PBS and with the incubated media containing vehicle or DBM. Duplicate sets of transfected cells were separated and incubated under normoxia or hypoxia, as described above, for 16 h. Luciferase activity was determined using a commercial kit TROPIX (Bedford, MA) by a BMG Labtechnologies LUMIstar Galaxy luminometer. Luciferase activity was expressed in arbitrary units. Protein was determined by the BCA method (Pierce, Rockford, IL).

Ubiquitination assay. A plasmid was constructed containing p3xFLAG-myc-CMV-25 (Sigma) with a coding sequence HIF-1α cDNA inserted into the *Hin*dIII/*Not*I site. HEK cells were transiently transfected with the plasmid for 24 h and then treated with vehicle, 250 μM $CoCl_2$, 100 μM DBM, or 10 μM MG-132. After 4 h the cells were collected and lysed in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100. Immunoprecipitation was performed with anti-FLAG M2 affinity gels (Sigma). The samples were boiled in SDS sample buffer and analyzed by SDS–PAGE. Western blots were developed with antibody to ubiquitin and anti-HIF-1α.

Data analysis. The data presented present representative data from three or more experiments. Quantification of densities was performed by NIH Image (version 1.62). Statistical analysis was performed using a one-way ANOVA test ($P < 0.05$).

Results

DBM increases HIF-1α and VEGF levels

We first studied the effect of DBM on HIF-1α protein in a prostate cancer cell line, PC-3. The cells were treated with DBM for 4 h at concentrations of DBM up to 100 μM. Under conditions of normal oxygen tension HIF-1α protein levels in the nuclear fractions were low, but were dramatically increased by the addition of DBM (Fig. 1A, left). HIF-1α was detected at about 20 μM DBM (Fig. 1, lower panel) and increased with increasing concentration up to the highest levels utilized. Under hypoxic conditions HIF-1α was induced in high levels in the absence of DBM (Fig. 1A) and these levels were only

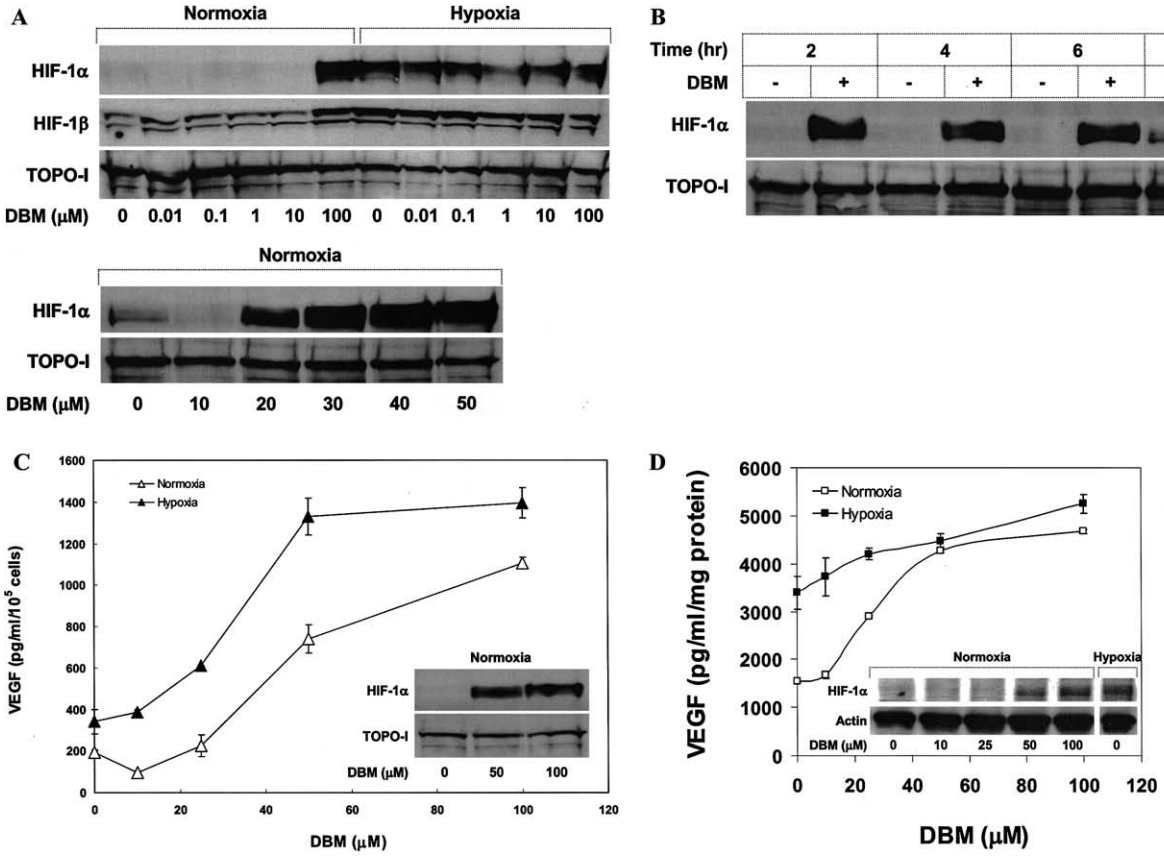

Fig. 1. DBM increases expression of HIF-1α and secretion of VEGF in prostate cancer cells. (A) PC-3 cells were treated with vehicle (0.1% DMSO) or with DBM at the indicated concentration (all contained 0.1% DMSO) for 4 h. NEs were prepared and Western blots were performed developed with antibody to HIF-1α. (B) PC-3 cells were treated with 50 μM DBM for the indicated times. The cells were then harvested and NEs were prepared for Western blotting with antibody to HIF-1α. The blots were stripped and re-probed with HIF-1β and TOPO-I. (C) LNCaP cells were treated with DBM at the indicated concentration of DBM under normoxic and hypoxic conditions for 4 h. The media from the above incubations were analyzed for VEGF expressed as pg/ml per the total number of cells in each well. The inset shows a Western blot of NEs of selected samples. (D) Neonatal primary cultures of cardiomyocytes from rat were prepared (see "Materials and methods"), treated with DBM as in (C). The media from the above incubations were analyzed for VEGF expressed as pg/ml per the total amount of protein in each well. The inset shows a Western blot of WCEs of samples.

insignificantly increased by the addition of DBM. The levels of HIF-1α in the nucleus under normoxia with DBM equaled or exceeded the maximum induction by hypoxia. There was a slight effect increasing HIF-1β by DBM, or by hypoxia in PC-3 cells, but the effect was not as large as for HIF-1α. Consistent with earlier results from our laboratory HIF-1β and HIF-1α shared common signaling pathways for nuclear protein accumulation [31]. A near maximal increase in HIF-1α under normoxia occurred rapidly, in less than 2 h (Fig. 1B), and levels began to decrease after 12 h.

Since PC-3 cells expressed relatively low detectable levels of VEGF protein we used another prostate cancer cell line, LNCaP cells. Treatment of LNCaP cells with DBM resulted in a similar stimulation of nuclear HIF-1α protein levels (Fig. 1C, inset). Conditioned media from the same treated cells showed a commiserate increase in VEGF protein levels upon treatment with DBM under normoxic conditions (Fig. 1C, open triangles). The half maximum response at about 40–50 μM was about the same level as the half maximum response of the HIF-1α protein (Fig. 1C, inset). Under hypoxic conditions (Fig. 1C, filled triangles) in the absence of DBM there was an expected increase in VEGF protein levels in the media, which was further augmented by increasing amounts of DBM. At the maximum response the levels attained by DBM treated normoxic cells approached those seen in treated hypoxic cells, and the levels in treated normoxic cells were 3-fold greater than those obtained in untreated hypoxic cells. High concentrations of DBM resulted in higher levels of VEGF than did hypoxia alone and the effect of the combination of DBM and hypoxia was additive. While DBM treatment under hypoxia resulted in a small increase in HIF-1α, this was disproportionately reflected in a larger increase in VEGF.

We further extended our experiments and tested the effect of DBM on VEGF and HIF-1α in non-cancerous cells. Primary culture of cardiomyocytes from rat hearts was prepared and treated with increasing doses of DBM under normoxic and hypoxic conditions (Fig. 1D).

Similar to LNCaP cells, VEGF levels in the media were increased in a dose-dependent manner under both normoxia and hypoxia (Fig. 1D). The HIF-1α protein was also induced after DBM treatment under normoxia (Fig. 1D, inset). DBM was able to induce nuclear HIF-1α protein and increased the production of VEGF in cancerous and non-cancerous cells.

DBM increases HIF-1 transcriptional activity

To test whether the induced HIF-1α protein was functional, we measured HIF-1 transcriptional activity using a reporter gene assay. HEK 293 cells were transiently transfected with a reporter plasmid containing *luciferase* gene under the control of hypoxia response element from the *VEGF* promoter and subsequently were treated with DBM (Fig. 2). As expected, there was almost no luciferase activity under normoxia while with increasing concentration of DBM there was a dose-dependent increased luciferase activity (Fig. 2A). This corresponded to increased HIF-1α levels (Fig. 1A, inset). The luciferase activity was induced under hypoxia and with the addition of DBM there was a modest increase in the activity (Fig. 2B). This was similar to what has been shown on protein level of HIF-1α (data not shown). This result showed that DBM induced a functional HIF-1α protein and the induction of VEGF by DBM was most likely driven by the activated HIF-1.

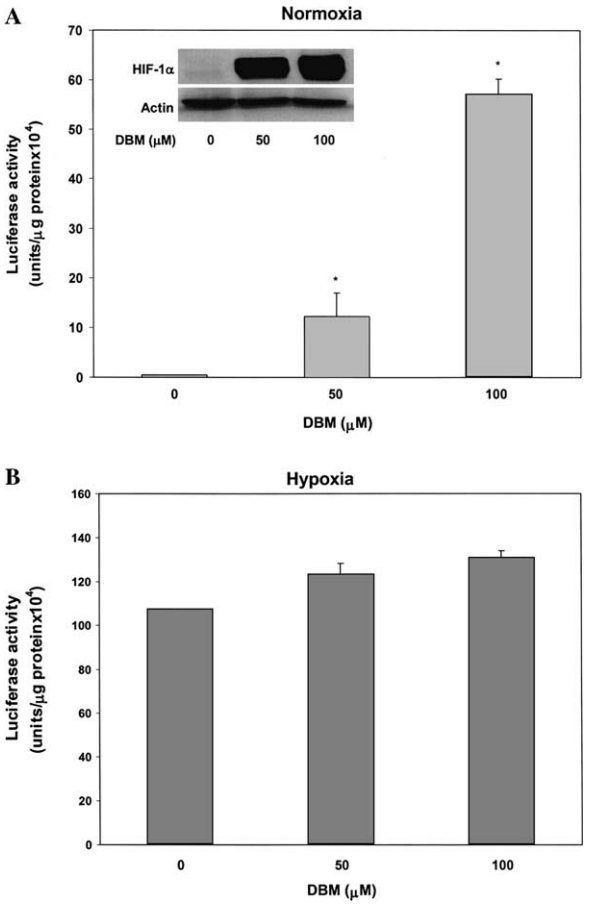

Fig. 2. DBM induces HIF-1α protein and HIF-1 transcriptional activity in HEK 293 cells. HEK 293 cells transiently transfected with pBI-GL V6L were treated with vehicle or DBM under normoxic (A) and hypoxic (B) conditions. Luciferase reporter activity was measured in the whole cell extract 16 h later. Relative luciferase activity represents units per microgram protein in each assay point. The inset in (A) shows a Western blot of whole cell extracts of the samples treated under normoxia that was developed with anti-HIF-1α and anti-actin. Columns, means; bars, SD; $n = 3$; *, $P < 0.01$.

DBM is uniquely powerful in increasing HIF-1α expression

The major regulation of HIF-1 activity involved stabilizing the HIF-1α subunit under hypoxia. It has been shown previously that in addition to hypoxia other stimulators such as $CoCl_2$ and iron chelators could also induce HIF-1α protein and activate HIF-1 [23]. To compare the effect of DBM to other HIF-1α stimulators, we treated HEK 293 cells with $CoCl_2$, the iron chelator desferroximine (DFO), and the proteasome inhibitor MG-132 as well as with hypoxia (Fig. 3A). Compared to

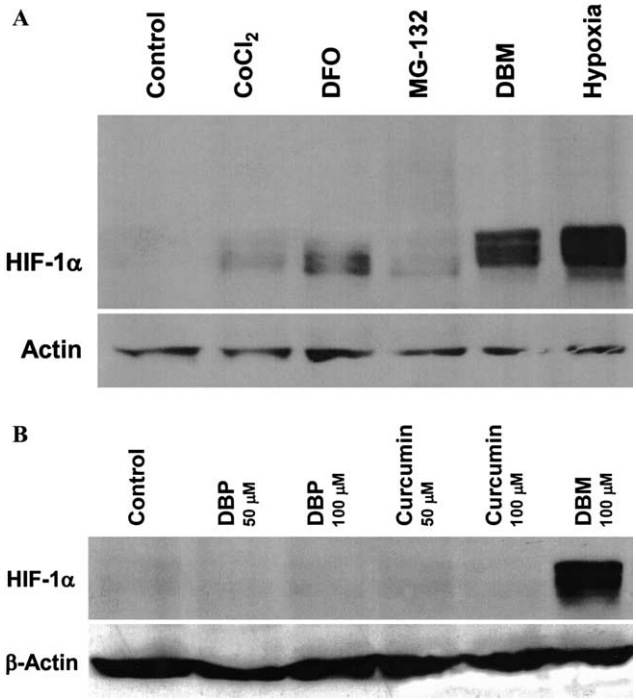

Fig. 3. Comparison of DBM-induced HIF-1α with other HIF-1α stimulants and other DBM analogs. (A) HEK 293 cells were treated with vehicle, 250 μM $CoCl_2$, 150 μM DFO, 10 μM MG-132, or 50 μM DBM for 4 h under normoxic conditions or subjected to hypoxia for 4 h. (B) HEK 293 cells were treated with vehicle, DBP, curcumin, or DBM at the indicated concentration for 4 h. The cells were then harvested and whole cell extracts were prepared for Western blotting with anti-HIF-1α and re-blotting with anti-actin antibodies. Note that the film was exposed to optimally illustrate induction of HIF-1α under hypoxia, $CoCl_2$ induced HIF-1α is still significantly increased compared with DMSO vehicle control.

control, all stimulants increased HIF-1α protein levels on Western blot under normoxic conditions. With MG-132 treatment, higher molecular weight immunoreactive species were also observed in addition to HIF-1α protein band (Fig. 3A, lane 4). Since MG-132 inhibited proteasomal degradation at the time the ubitquitinylation process continued to occur, it was probable that these bands corresponded to the poly-ubiquitin conjugates of HIF-1α (Fig. 3A, lane 4). In addition, we compared the effect of DBM to other DBM analogs (Fig. 3B). All the DBM analogs used in this experiment were not effective. There was no increase in HIF-1α levels by DBP (1,3 dibenzoyl propane) or curcumin. DBP has three methylene ($-CH_2-$) groups between the benzoyl radicals ($C_6H_5C{=}O$) unlike DBM (1,3 diphenyl 1,3 propane dione), which has one methylene group. Curcumin (1,7 bis (4-hydroxy-3-methoxphenyl-1,6-heptadione-3,5-dione)) had the same $R{-}(C{=}O){-}CH_2{-}(C{=}O){-}R$ as DBM but instead of direct linkage to an aromatic ring, the dione was linked by an ethylene group to a derivativized phenol. These results suggest that the effect of DBM on HIF-1α was restricted to a specific structure present in DBM.

DBM decreases the rate of HIF-1α protein degradation

To analyze the effect of DBM on HIF-1α protein, we used the protein translation inhibitor, cycloheximide (CHX) (Figs. 4A and B). In the presence of CHX new protein synthesis was inhibited and HIF-1α levels would predominantly reflect the degradation process of HIF-1α protein. We treated HEK 293 cells with either vehicle or DBM and subsequently performed a time course of HIF-1α protein disappearance in the presence of CHX (Fig. 4A). In cells without DBM the HIF-1α protein levels dropped rapidly especially the lower part of the band (Fig. 4A, left panel). In contrast, the DBM treated cells showed the anticipated increase in HIF-1α, which was stabilized and persisted until greater than 30 min (Fig. 4A, right panel). Quantitative densitometry of the total (upper and lower parts) HIF-1α immunoreactive bands showed the rapid decline of HIF-1α in the absence of DBM compared with stabilization of HIF-1α in the presence of DBM (Fig. 4B). There was a 50% decrease in the HIF-1α levels in the untreated cells by 8 min (Fig. 4B, closed triangles), whereas in the treated cells a 50% decrease was not observed until 60 min (Fig. 4B, open triangles). To further understand at what step DBM affects HIF-1α, we studied HIF-1α ubiquitinylation in the presence or absence of DBM and directly compared it with $CoCl_2$ and MG-132. HEK 293 cells were transiently transfected with a plasmid coding for a FLAG fused HIF-1α. Antibody to FLAG would immunoprecipitate HIF-1α with varying degrees of ubiquitination, whereas antibody to HIF-1α has different

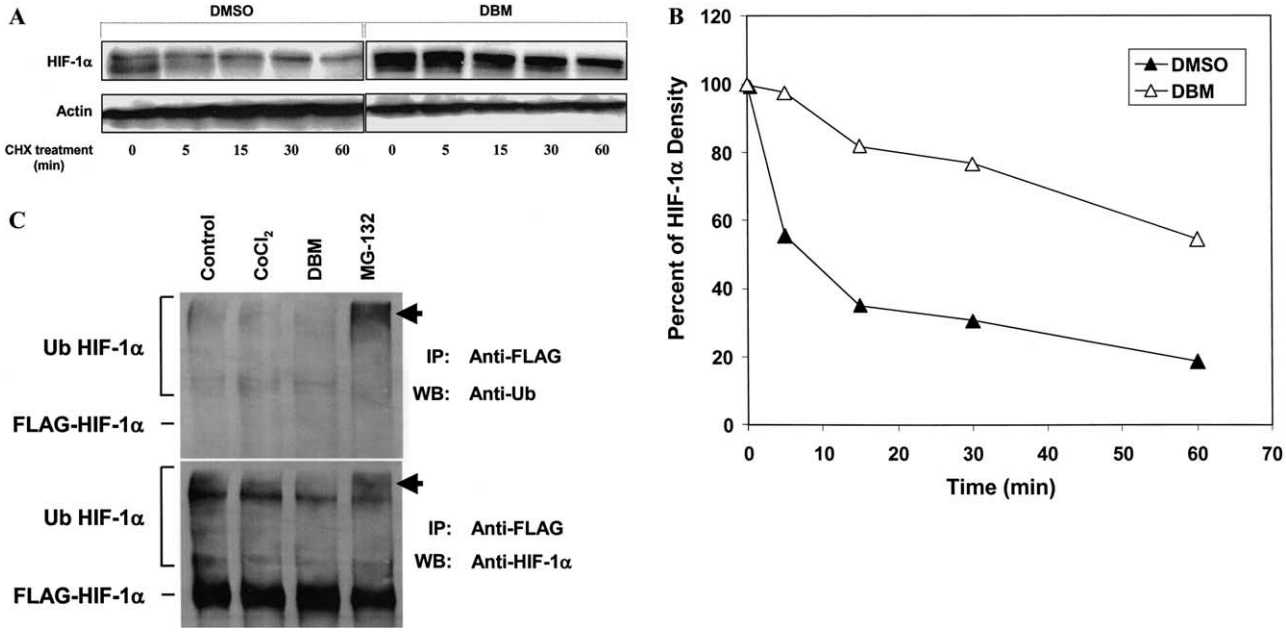

Fig. 4. DBM extends half-life and reduces ubiquitination of HIF-1α protein. (A) HEK 293 cells were treated either with vehicle (0.05% DMSO) or 50 μM DBM under normoxic conditions. After 4 h, 100 μM of CHX was added and the cells were incubated for the indicated time (min). The cells were harvested, whole cell extracts were prepared, and Western blots were developed with anti-HIF-1α and then re-blotted with anti-actin antibodies. The Western blots of treated and untreated samples presented are of different exposure times to enable clearer visibility. (B) Represents densitometric quantification of HIF-1α band from each time point of (A). (C) HEK 293 cells transiently transfected with p3xFLAG-HIF-1α were treated with vehicle, 250 μM $CoCl_2$, 100 μM DBM, or 10 μM MG-132 for 4 h. The cells were then harvested, whole cell extracts were prepared, and immunoprecipitation (IP) was performed with anti-FLAG antibody as described under "Materials and methods." The immunoprecipitates were resolved on SDS–PAGE, analyzed by Western blot (WB) with ubiquitin (Ub), and then re-probed with HIF-1α antibodies. The arrow indicates accumulation of poly-ubiquitinated forms of HIF-1α.

affinities to different species of poly-ubiquitinated HIF-1α protein. The use of the FAG labeled HIF-1α reflects the distribution of the HIF-1α more faithfully than the native antibody. Immunoprecipitates using antibody to the FLAG portion were prepared from cells treated with vehicle, $CoCl_2$, DBM, or the proteasome inhibitor MG-132.The solubilized immunoprecipitates were electrophoresed and the Western blot was developed with antibody to ubiquitin (Fig. 4C, upper panel). Accumulation of the ubiquitinated form of HIF-1α would have been anticipated if DBM had blocked steps after the ubiquitination. In the presence of DBM there was no increase in the amount of ubiquinated HIF-1α compared to control, indicating that DBM blocked the degradation prior to the ubiquinitation step. On the other hand, the proteasome inhibitor MG-132, which blocks degradation after the ubiquitination step, resulted in the accumulation of significant levels of ubiquitinated HIF-1α. In particular the higher molecular weight, multiple ubiquitinated adducts were observed (Fig. 4C, upper panel, lane 4). Similar to DBM, $CoCl_2$ also did not result in increased ubiquinated forms of HIF-1α protein. Development of the Western blots with anti-HIF-1α confirmed that the anti-FLAG antibody indeed precipitated the HIF-1α protein (Fig. 4C, lower panel). Again there was no detected increase in the ubiquitinated HIF-1α (Fig. 4C, lower panel, lane 3). Taken together, these results demonstrated that DBM decreased the rate of HIF-1α degradation at a step prior to ubiquitination and thereby activating HIF-1 and its downstream targets including VEGF.

Discussion

Most conventional iron chelators used for clinical and experimental iron chelation, as a side effect, lead to the activation of HIF-1 and its downstream responses [32–34]. In this manuscript we have described the effect of the iron chelator, DBM, on HIF-1 pathway in vitro. DBM is a nontoxic phytochemical found in low levels in licorice plants, which may be a carcinogen antagonist [35–39].

We found that DBM induced an increase in the levels of HIF-1α protein in prostate cancer cells (PC-3 and LNCaP), in HEK 293 (human embryonic kidney) cells, and primary cultured cardiomyocytes (Fig. 1). DBM treatment resulted in a dramatic increase in transcriptionally active HIF-1 and a concomitant rise in the levels of VEGF. Moreover, we found that the induction by DBM occurs in normoxic cells through the inhibition of HIF-1α protein degradation (Fig. 4). The rapid degradation of HIF-1α protein through the proteasome pathway under normoxia was mediated by a prior prolyl hydroxylation mechanism at proline residues 564 and 402 [10–12]. This hydroxylation process required specific HIF-prolyl hydroxylases and the presence of iron and oxygen [13,14]. Since iron was required for the proline hydroxylation step, we believed that lack of this enzymatic activity in the presence of iron chelators such as DFO and DBM resulted in the increased levels of HIF-1α under normoxia. However, not all iron chelators could activate the HIF-1 response pathway. Curcumin [40] and the iron chelator, HBSer (*N*-(2-hydroxybensyl)-L-serine) [41] did not increase HIF-1α.

DBM was biologically available when fed to animals and has been used as a cancer protective agent in mice [42,43]. The mechanism for this effect is not clear. On the one hand it may be that DBM interferes with the mechanism of carcinogenesis by the anthracene-based carcinogens used in these studies, perhaps by iron chelation and inhibition of oxygen free radical formations. On the other hand, overproduction of HIF-1α in some cells has been associated with induction of apoptosis and cell death [24]. The oral bioavailability and low toxicity of DBM make it tempting to suggest its use in inducing angiogenesis in vivo where this process could be applied to reverse ischemic diseases. Future studies to understand the exact mechanism of DBM action of HIF-1 pathway and its effect in in vivo systems are warranted.

Acknowledgments

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