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Honokiol inhibits HIF pathway and hypoxia-induced expression of histone lysine demethylases

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ABSTRACT

Hypoxia-inducible-factor (HIF)-mediated expression of pro-angiogenic genes under hypoxic conditions is the fundamental cause of pathological neovascularization in retinal ischemic diseases and cancers. Recent studies have shown that histone lysine demethylases (KDMs) play a key role in the amplification of HIF signaling and expression of pro-angiogenic genes. Thus, the inhibitors of the HIF pathway or KDMs can have profound therapeutic value for diseases caused by pathological neovascularization. Here, we show that hypoxia-mediated expression of KDMs is a conserved process across multiple cell lines. Moreover, we report that honokiol, a biphenolic phytochemical extracted from *Magnolia* genus which has been used for thousands of years in the traditional Japanese and Chinese medicine, is a potent inhibitor of the HIF pathway as well as hypoxia-induced expression of KDMs in a number of cancer and retinal pigment epithelial cell lines. Further, treating the cells with honokiol leads to inhibition of KDM-mediated induction of pro-angiogenic genes (adrenomedullin and growth differentiation factor 15) under hypoxic conditions. Our results provide an evidence-based scientific explanation for therapeutic benefits observed with honokiol and warrant its further clinical evaluation for the treatment of pathological neovascularization in retinal ischemic diseases and cancers.

1. Introduction

HIF, the master regulator of neovascularization, is a transcription factor that plays vital roles during oxygen deficiency (*i.e.* hypoxia) [1]. The active HIF is a heterodimeric protein and consists of two subunits, HIF-α and HIF-1/ARNT. Transcriptional regulation by oxygen is mediated by the HIF-α isoforms. In humans, three isoforms of α-subunit (HIF-1α, HIF-2α, and HIF-3α) have been identified. HIF-1α and HIF-2α are closely related and each possess two conserved sites of prolyl hydroxylation (Pro402, Pro564 in human HIF-1α) [2,3]. Prolyl hydroxylation at these positions by the oxygen-dependent HIF prolyl hydroxylases (PHD1-3) allows the binding of HIF-α isoforms with the von Hippel-Lindau tumor suppressor (pVHL) followed by their proteosomal degradation under normoxia [4,5]. HIF-1α and HIF-2α also possess a highly conserved asparagine residue (Asn803 in HIF-1α, which is hydroxylated by FIH in humans) that prevents its interaction with the transcriptional co-activator p300 under normoxia [2,3]. Under hypoxia, PHDs become inactive, allowing HIF-α to escape prolyl hydroxylation and proteosomal degradation. Subsequently, HIF-α translocates to the nucleus, dimerizes with HIF-1β and recruits several transcriptional co-activators. Thus, during hypoxia, the active HIF-α/β heterodimer binds to a core DNA sequence (G/ACGTG) in the hypoxia-response-element (HRE) present in the promoters of target genes causing overexpression of VEGF and other pro-angiogenic factors.

Recent studies suggest that transcriptional adaptation to hypoxia also involves epigenetic changes in the histone methylation [6,7]. The HIF pathway upregulates, in addition to many known proteins that facilitate metabolic adaptation to hypoxia, transcription of a number of Jumonji-C (JmjC) domain containing iron (II), 2-oxoglutarate (2OG)-dependent histone lysine demethylases (KDMs) in the hypoxic cancer cells [8]. Of these KDMs, four (*JMJD1A, JMJD2B, JMJD2C* and *JARID1B*) are direct transcriptional targets of the HIF pathway as HIF-α/β heterodimer binds to the HRE present in the promoters of these genes [8]. JMJD1A (also known as *KDM3A* or *JHDM2A*) regulates the expression of some hypoxia-inducible pro-angiogenic genes (adrenomedullin, *ADM* and growth differentiation factor 15, *GDF15*) by decreasing the histone methylation levels at their promoters, and knock-down of JMJD1A

Abbreviations: JmjC, jumonji-C domain; KDM, histone lysine demethylase; 2OG, 2-oxoglutarate; VEGF, vascular endothelial growth factor; HRE, hypoxia response element; PHD, prolyl hydroxylation domain; VHL, von hippele-lindau.

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reduces the tumor growth [8,9]. Consistently, we have recently reported that hypoxia also induces the expression of KDMs in retinal pigment epithelial cells [10]. Moreover, our studies showed that the expression of pro-angiogenic genes (*ADM*, *GDF15*, *HMOX1*, *SERPE1* and *SERPB8*) is dependent on KDMs under hypoxic conditions and treating the cells with a general KDM inhibitor blocks their expression.

Hypoxia is experienced in many pathological conditions like cancer, ischemic heart diseases, stroke, diabetic retinopathy, glaucoma etc. Strong evidences have established that the expression of pro-angiogenic factors (*e.g.* VEGF etc.), which play critical role in pathological neovascularization in cancer and eye diseases, is elevated due to the activation of HIF pathway under hypoxia [11,12]. Current attempts to inhibit pathological angiogenesis in the retinal ischemic diseases and cancers, mostly use inhibitors of one or two growth factors and/or signaling intermediates. This strategy has had only partial success, and most relevant clinical trials have shown modest effects thus far. The relative inefficiency of inhibitors of single targets may be due to the multifactorial and temporal nature of the angiogenic process. Thus, possible future approaches to successfully fight pathological neovascularization may rely either on blocking some master modulators, such as HIF or KDMs (which amplify the HIF signaling), or on a combination therapy inhibiting several targets simultaneously. In this article we report that honokiol inhibits the HIF pathway and hypoxia-induced expression of KDMs in a number of cancer and retinal pigment epithelial cells. Further, treating the cells with honokiol leads to inhibition of KDM-mediated induction of pro-angiogenic genes under hypoxic conditions. The results from this study identify an important molecular target of honokiol and provide an evidence-based scientific explanation of its therapeutic benefits.

2. Materials and methods

2.1. Cell culture

All the cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA), except the human retinal pigment epithelial cells (D407), which were generously gifted by Dr. Richard Hunt (University of South Carolina). Cells were grown in DMEM medium supplemented with 20 mM HEPES, 29 mM sodium bicarbonate, 1% nonessential amino acids, 10% heat inactivated FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin (media components were from Sigma Chemical Co., St. Louis, MO). Cells were maintained at 37 °C, 5% CO_2 and 90% relative humidity. The medium was changed every 2–3 days. After reaching ≈90% confluency, cells were detached using TrypLE™ Express (Invitrogen, Grand Island, NY) and were passaged. Cells were seeded at a density of 1 million cells in 25cm^2 flasks and used for further studies at ≈70% confluency. Honokiol (LKT laboratories, St. Paul, MN) was dissolved in DMSO at 12.49 mg/ml concentration. Inhibitor studies were carried out by adding varying concentrations (5, 10, 20 μM) of honokiol to the culture media. In the control samples 0.04% of DMSO, corresponding to the DMSO concentration in the highest honokiol treatment, was added.

2.2. Hypoxia treatment

Cells were treated to hypoxic condition in a Bactron anaerobic chamber (Sheldon Manufacturing, Cornelius, OR). An artificial gas mix of 1% O_2, 5% CO_2, and 94% N_2 was used to maintain hypoxia in the chamber. The chamber was purged in order to generate a hypoxic atmosphere, following the manufacturer's instructions. Partial pressure of oxygen inside the chamber was confirmed to be 1% by using dissolved oxygen meter (Extech Instruments, Nashua, NH). Upon reaching ≈70% confluency, cells were aseptically transferred into the hypoxic chamber through the airlocks in order to maintain the hypoxic conditions inside the chamber. D407 cells were incubated at 37 °C under hypoxic conditions for 24 h as per our earlier publication [10]. All other cell lines were exposed to 12 h of hypoxia because maximal induction of hypoxic response genes was observed at that time point. Cell lysis for RNA extraction was performed inside the hypoxia chamber in order to avoid any exposure of the cells to normoxic conditions.

2.3. RNA extraction from cells

Cells grown under normoxic or hypoxic conditions, in the presence or absence of honokiol, were lysed with TRI® reagent (Molecular Research Center, Inc., Cincinnati, OH). The lysate was collected into a micro centrifuge tube and treated with chloroform for phase separation. The aqueous phase containing RNA was separated and 0.7 volumes of isopropanol (100%) was added to precipitate RNA. After two 75% ethanol washes RNA pellet was dissolved in DNase/RNase-free water. The RNA concentration was measured using Nanodrop (Thermo Fisher Scientific, Wilmington, DE) and 6 μg of RNA was reverse transcribed using M-MLV reverse transcriptase as per manufacturer instructions (Promega, Madison, WI).

2.4. Quantitative real time PCR analysis

Quantitative real time PCR (qPCR) of all the samples were performed as described earlier [10]. Briefly, 20 μl qPCR reactions were performed with 2.5 units of Taq polymerase (Bulldog Bio, Inc., Portsmouth, NH), 0.25 mM dNTP (Fisher Scientific, Hanover Park, IL), 1X SYBR (Invitrogen) green, 80 ng cDNA and 1.5 μM of forward and reverse primers. The qPCR reactions were performed in biological triplicates and in experimental duplicates using a LightCycler® 480 qPCR instrument (Roche Diagnostics Corporation, Indianapolis, IN). Ribosomal protein L32, β-actin, and GAPDH were used as internal controls to obtain ΔC_t. The ΔC_t values of the treated samples were normalized with its corresponding untreated ΔC_t values to give $\Delta\Delta C_t$. The relative fold change in the expression levels of the genes is represented by $2^{-\Delta\Delta Ct}$ value. The primer sequences used for this study are listed in supplementary materials (Table S1).

2.5. LCMS analysis of honokiol

Purity of honokiol was evaluated by LCMS analysis using an Agilent Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 3.5 μm particle size) column. ABI 3200 Q-Trap mass spectrometer fitted with electrospray ionization (ESI) source interfaced to a Shimadzu HPLC system equipped with a diode array detector (DAD) was used for LCMS analysis. A 20 min water and acetonitrile (ACN) gradient method was run at a flow rate of 0.5 ml/min with the following parameters: 0–2 min at 5% ACN, 5% to 95% ACN in the next 4 min and holding at 95% for another 4 min, then dropping to 5% ACN in the next 8 min and holding for 2 min at 5% ACN. Honokiol was detected by setting the DAD to 280 nm. The detection in the mass spectrometer was done in the positive mode using EMS full scan.

2.6. Cytotoxicity assay

Cytotoxicity was determined using MTS assay at different concentrations of honokiol. Cells were plated at a density of 10,000 cells/well in 96-well plate. After reaching 50–60% confluency, cells were exposed to varying concentrations of honokiol under normoxic or hypoxic conditions. The media was replaced with fresh 100 μl of serum free media and Cell Titer 96® Non-Radioactive cell proliferation assay kit (Promega) was employed according to

the manufacturer's protocol. After incubating for 4 h, the quantity of formazan formed (which is proportional to the number of viable cells) was measured at 490 nm using a 96-well micro titer plate reader (Spectra Fluor Plus, Tecan, Maennedorf, Switzerland).

3. Results

3.1. Hypoxia-mediated expression of KDMs is a conserved process

Recent studies have shown that hypoxia induces the expression of a number of KDMs in different cancer and retinal pigment epithelial cells, suggesting a new layer of epigenetic transcriptional regulation of hypoxia response genes [6–8,10]. Although, the HRE is present in the promoters of four KDMs (*JMJD1A, JMJD2B, JMJD2C* and *JARID1B*) [8], these studies have indicated that hypoxia can induce the expression of any one to all four KDMs depending on the cell lines. Therefore, we verified the expression of these KDMs and selected markers of the HIF pathway (HIF-1α, HIF-2α, HIF-1β, VEGF and GLUT) under hypoxic conditions in human retinal pigment epithelial cells (D407), human colon adenocarcinoma grade II cells (HT29), human breast adenocarcinoma cells (MCF7), and human embryonic kidney cells (HEK293). The cDNA from D407, HT29, MCF7, and HEK293 cells, grown under hypoxic (1% O_2) or normoxic (21% O_2) conditions, as described in the materials and methods section; were synthesized and utilized for expression analysis by qPCR. Two independent primer pairs from different regions of the same gene were employed except for a few genes where only one validated primer pair was used. The C_t values were normalized with their respective internal controls. Since a recent report has shown that the levels of expression can vary between hypoxic and normoxic conditions depending on the internal control [13], we have exploited three different internal controls (ribosomal protein L32, β-actin, and GAPDH) to normalize the C_t values. The fold changes were calculated by comparing the hypoxic samples to their normoxic counterparts. To this end, based on the three different internal controls no significant changes in the expression levels were observed in any of the four cell lines used in this study (data not shown); therefore, subsequent results are presented based on normalization with the ribosomal protein L32.

Consistent with previous reports, we observed an up regulation of the known HIF pathway markers (GLUT and VEGF) under hypoxic conditions in all the cell lines (Fig. 1). These experiments further demonstrated that the expression of all four JmjC domain containing iron (II), 2OG-dependent KDMs, that are direct transcriptional target of HIF [8], also peaked within the same time frame irrespective of the origin of the cell lines (Fig. 2). Although the expression levels of KDMs varied, depending on the cell line and the duration of the hypoxia treatment (data not shown), our studies indicated that *JMJD1A* was one of the strongly induced KDMs under hypoxic conditions across most cell lines. *JMJD1A* acts as a co-activator for nuclear hormone receptors and regulates transcription by demethylating di- and mono-methylated histone-3 lysine-9 (H3K9-me2 and H3K9-me) into H3K9, at target promoters [14]. Since recent studies have indicated that the expression of a subset of hypoxia induced genes (*e.g. ADM* and *GDF15*, which play critical roles in HIF signaling and angiogenesis [15–17]) is regulated by *JMJD1A* [8,10], we further investigated the expression of these two *JMJD1A* targets. After hypoxia treatment, significant induction was noted for these downstream targets of *JMJD1A* compared to normoxic conditions (Fig. 2). These results confirm that hypoxia-mediated induction of KDMs and JMJD1A targets is a conserved process across multiple cell lines.

3.2. Honokiol Inhibits HIF pathway

An activation of the HIF pathway leading to hypoxia-induced neovascularization is the central cause of pathogenesis of almost all solid tumors and ischemic retinal diseases [1,11,12]. These studies suggest that specific inhibitors of the HIF pathway or KDMs can be used as a new therapeutic approach to manage diseases caused by hypoxia. Therefore, in order to identify inhibitors of the HIF pathway, we evaluated the effect of a number of known phytochemicals from traditional medicine. For these experiments, D407 cells were cultured in the presence or absence of

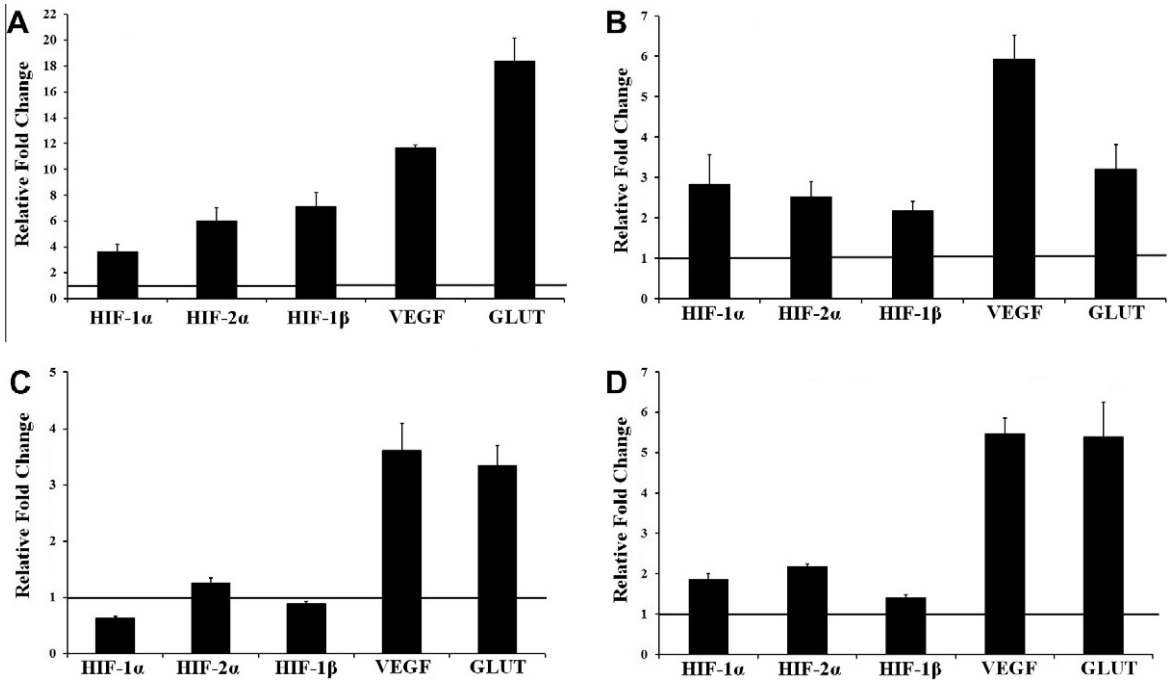

Fig. 1. Relative fold change in the mRNA levels of HIF isoforms and direct HIF targets (VEGF and GLUT) in various cell lines exposed to hypoxia: D407 (Panel A), HT29 (Panel B), MCF7 (Panel C), and HEK293 (Panel D). The bars represent relative mRNA fold change ± standard error. Normoxic levels are represented by the horizontal line.

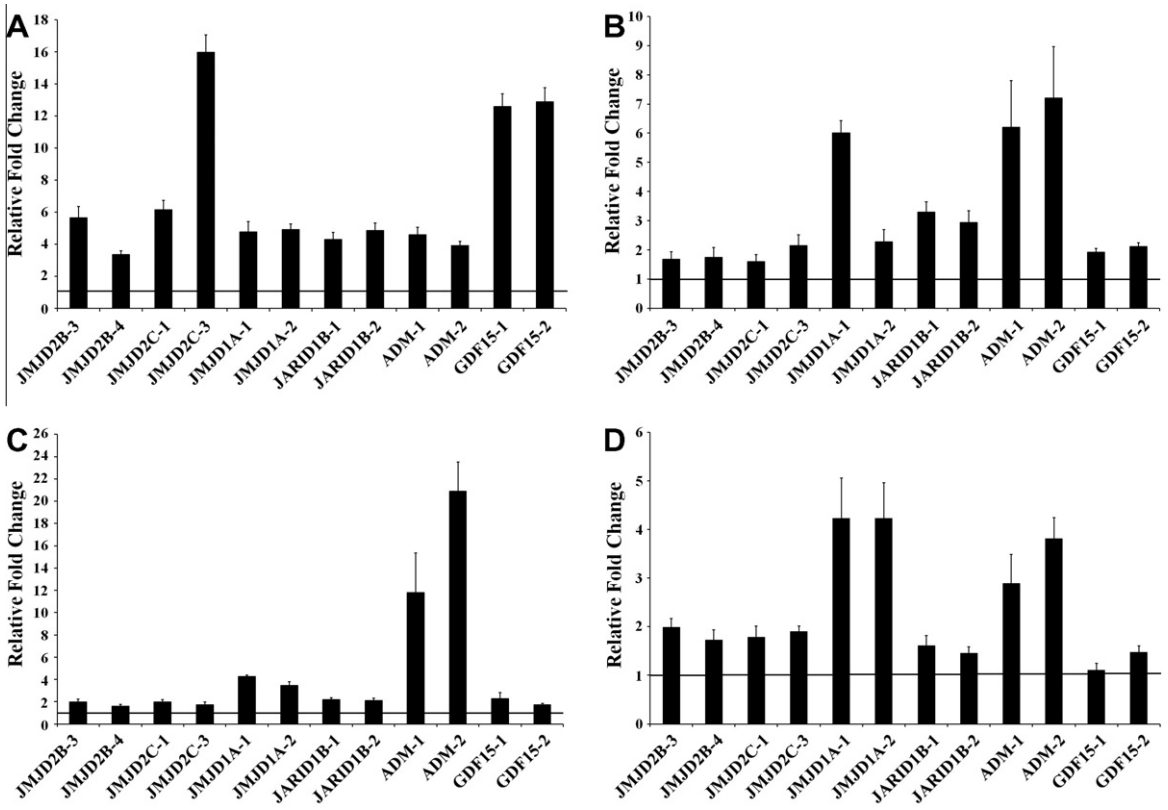

Fig. 2. Relative fold change in the mRNA levels of HIF regulated KDMs and JMJD1A direct targets (ADM and GDF15) in various cell lines exposed to hypoxia: D407 (Panel A), HT29 (Panel B), MCF7 (Panel C), and HEK293 (Panel D). The bars represent relative mRNA fold change ± standard error. Normoxic levels are represented by the horizontal line.

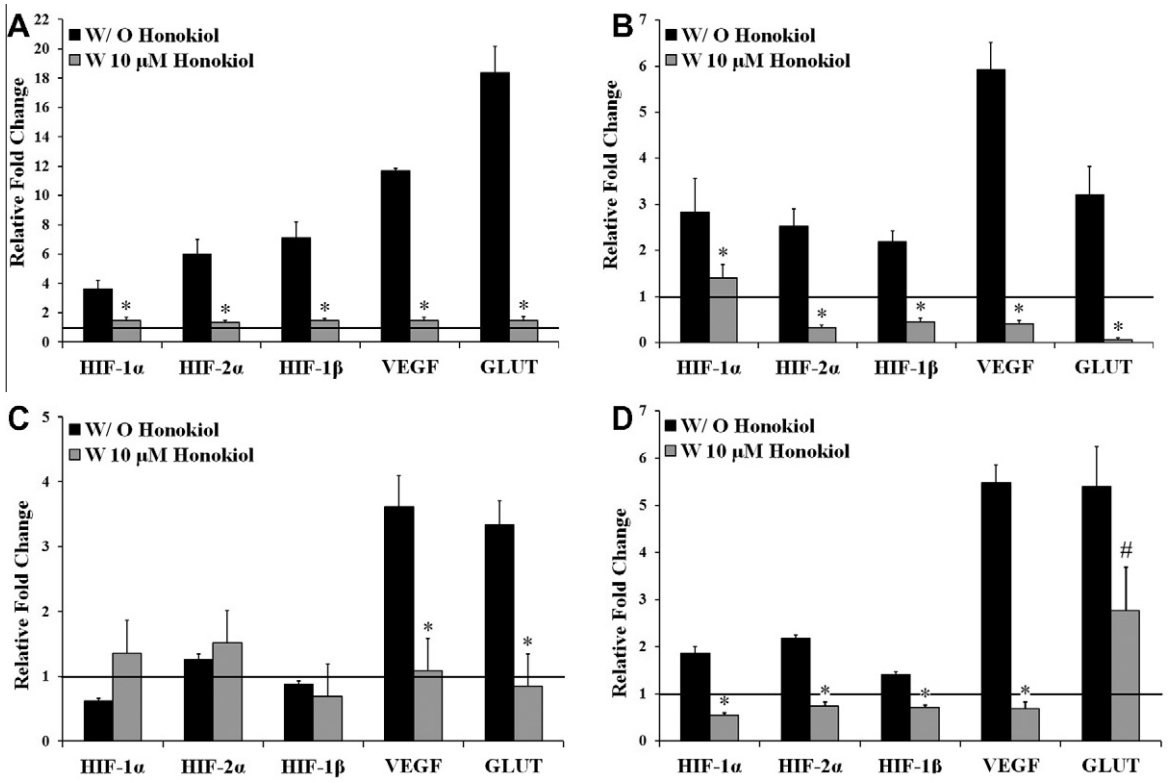

Fig. 3. Relative fold change in the mRNA levels of HIF isoforms and direct HIF targets (VEGF and GLUT) in various cell lines exposed to hypoxia: D407 (Panel A), HT29 (Panel B), MCF7 (Panel C), and HEK293 (Panel D) in presence or absence of 10 μM honokiol. Solid black and grey bars represent relative mRNA fold change ± standard error with or without honokiol, respectively. Normoxic levels are represented by the horizontal line. Student t-test assuming equal variance (one tailed) was performed. The treatment is significant when compared to the control at $P < 0.01$ represented by $^{*}P < 0.05$ represented by #.

phytochemicals under normoxic and hypoxic conditions for 24 h and the level of expression of HIF isoforms (HIF-1α, HIF-2α and HIF-1β) and hypoxia markers (direct HIF target genes, VEGF and GLUT) were compared. We found that honokiol, a biphenolic phytochemical extracted from *Magnolia* genus [18], strongly (≈50–90% for most genes) inhibited the expression of HIF isoforms and hypoxic markers at 5–20 μM concentrations in a concentration-dependent manner (Fig. S1). Since commercially available honokiol is generally purified from seed cones and/or bark of the *Magnolia* tree, we evaluated its purity using a LCMS method. This study showed a single LC peak at 10.85 min and its MS peak at 10.96 min with a mass of 266.2 amu corresponding to the molecular weight of honokiol (*Fig.* S2). This result demonstrates that inhibition of the HIF pathway is indeed due to honokiol and not because of any impurities present in the commercial product.

The studies with D407 cells indicated potent inhibition of the HIF pathway even at 10 μM of pure honokiol without any noticeable cytotoxicity both under hypoxic and normoxic conditions (Fig. S3). Therefore, we evaluated the effect of honokiol at this concentration on the HIF pathway in other cell lines (HT29, MCF7 and HEK293). These cell lines were cultured in the presence or absence of 10 μM of honokiol under normoxic and hypoxic conditions for 12 h, and the level of expression of HIF isoforms and hypoxic markers were compared. As with D407 cells, these experiments indicated that honokiol potently (≈40–90%) inhibited the expression of most HIF isoforms and hypoxia markers (Fig. 3).

3.3. Inhibition of hypoxia-mediated expression of KDMs and pro-angiogenic genes by honokiol

Recent studies have revealed that a number of KDMs, especially those that are direct HIF targets (*JMJD1A, JMJD2B, JMJD2C* and *JARID1B*), are also overexpressed in various cancers [19–23]. In renal cell carcinomas, higher levels of JMJD1A are present in hypoxic environment and around the blood vessels, suggesting its role in tumor angiogenesis [24]. JMJD1A regulates the expression of ADM and GDF15 by directly binding at their promoters [8,10], which play critical roles in HIF signaling and angiogenesis [15–17]. Taken together, these results further emphasize that inhibition of HIF-mediated expression of KDMs such as JMJD1A, which amplify HIF signaling, could lead to a new treatment for cancers and retinal ischemic diseases.

Therefore, the effect of honokiol on hypoxia-mediated expression of KDMs and pro-angiogenic genes was evaluated. Again for these studies we focused on KDMs that are direct transcriptional target of HIF, and ADM and GDF15, that are direct transcriptional target of JMJD1A. These experiments indicated that honokiol strongly (≈40–90% for most genes) inhibited the expression of KDMs and its target pro-angiogenic genes in D407 cells in a concentration-dependent manner (Fig. S4). Further, treating the other cell lines (HT29, MCF7 and HEK293) with 10 μM of honokiol indicated that honokiol potently inhibit (≈50–90%) the expression of most the HIF pathway intermediates and markers (Fig. 4). These results confirm that honokiol-mediated inhibition of the HIF pathway is conserved across multiple cell lines.

4. Discussion

Pathological activation of the HIF pathway leads to the expression of pro-angiogenic factors during the neovascularization in cancer and retinal diseases. Little is known about the epigenetic regulations associated with HIF-mediated transcription and activation of pro-angiogenic genes. Recent studies have shown that KDMs play a key role in the amplification of HIF signaling and expression of pro-angiogenic genes. In this study, we have proved that hypoxia-mediated expression of KDMs is a conserved process

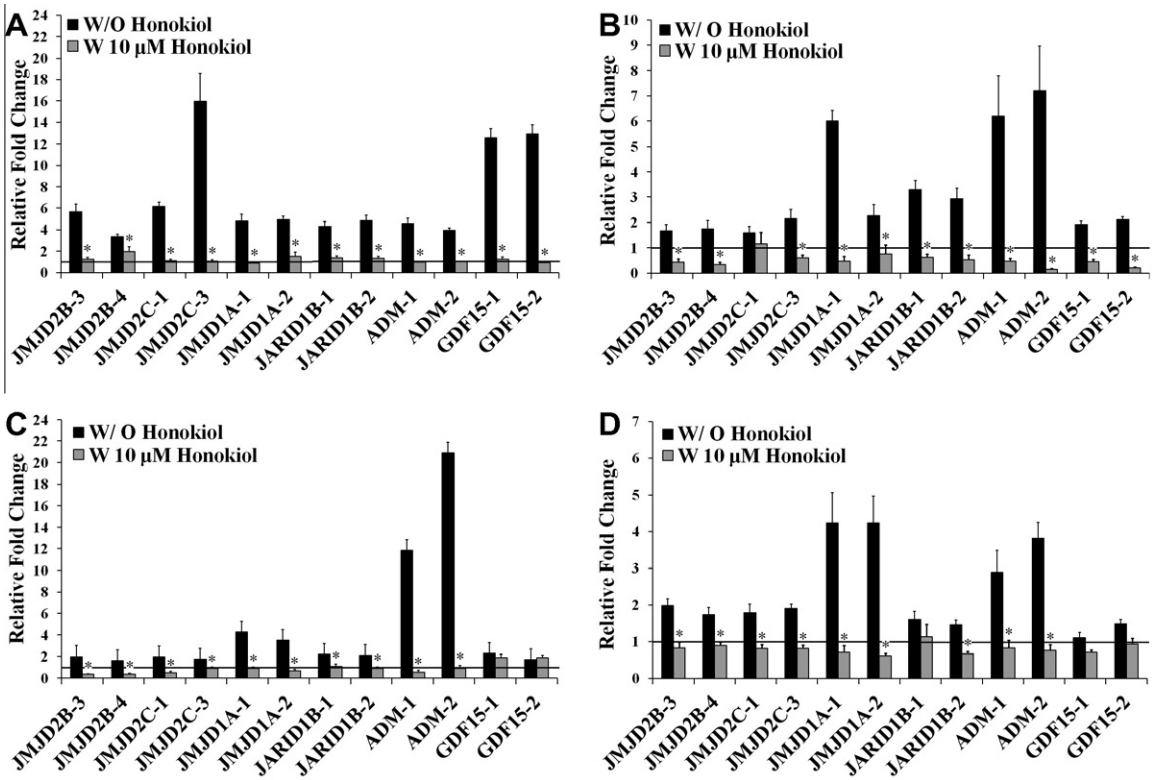

Fig. 4. Relative fold change in the mRNA levels of HIF regulated KDMs and JMJD1A direct targets (ADM and GDF15) in various cell lines exposed to hypoxia: D407 (Panel A), HT29 (Panel B), MCF7 (Panel C), and HEK293 (Panel D) in presence or absence of 10 μM honokiol. Solid black and grey bars represent relative mRNA fold change ± standard error with or without honokiol, respectively. Normoxic levels are represented by the horizontal line. Student t-test assuming equal variance (one tailed) was performed. The treatment is significant when compared to the control at $P < 0.01$ represented by ∗.

across multiple cell lines. Because inhibition of the HIF pathway or KDMs can have profound therapeutic value for diseases caused by pathological neovascularization, we screened a number of phytochemicals for their ability to inhibit this pathway. These studies identified honokiol as a potent inhibitor of the HIF pathway and hypoxia-mediated expression of KDMs in a number of cancer and retinal pigment epithelial cell lines. Further, treating the cells with honokiol leads to inhibition of KDM-mediated induction of pro-angiogenic genes under hypoxic conditions.

In traditional herbal medicines, honokiol has been used as a therapeutic agent with anti-angiogenic, anti-anxiety, anti-stroke, anti-tumor, and neuroprotective properties [18]. A number of recent studies have recapitulated these effects in preclinical models without noticeable toxicity [25]. These findings have generated interest in bringing honokiol to the clinic as a novel therapeutic agent. However, lack of comprehensive understanding of mechanism/s of honokiol-action has hindered this process. Interestingly, in a number of conditions, such as angiogenesis, stroke, neurodegeneration, etc., where honokiol is used as a therapeutic agent, pathological activation of the HIF pathway appears to play a major role in the manifestation of the disease [1]. Therefore, our results demonstrating inhibition of the HIF pathway and hypoxia-induced expression of KDMs by honokiol provides an evidence-based scientific explanation for its therapeutic benefits. Further, because the HIF pathway plays an important role in the development of cancer stem cells [26], which are highly resistant to conventional chemo or radiation therapy [27–29], honokiol may be combined with these therapies to improve the therapeutic response of cancer stem cells. Finally, HIF-mediated induction of pro-angiogenic genes, such as *VEGF* etc., are responsible for a number of ischemic retinal diseases such as VHL, retinopathy of prematurity, proliferative diabetic retinopathy, and glaucoma [11], our results warrant further clinical evaluation of honokiol for the treatment of pathological neovascularization in retinal ischemic diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.143.

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