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Flavonoids from Radix Astragali Induce the Expression of Erythropoietin in Cultured Cells: A Signaling Mediated via the Accumulation of Hypoxia-Inducible Factor-1α

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Supporting Information

ABSTRACT: Radix Astragali (RA) is commonly used as a health food supplement to reinforce the body vital energy. Flavonoids, including formononetin, ononin, calycosin, and calycosin-7-*O*-β-D-glucoside, are considered to be the major active ingredients within RA. Here, we provided different lines of evidence that the RA flavonoids stimulated the expression of erythropoietin (EPO), the central regulator of red blood cell mass, in cultured human embryonic kidney fibroblasts (HEK293T). A plasmid containing hypoxia response element (HRE), a critical regulator for EPO transcription, was tagged upstream of a firefly *luciferase* gene, namely, pHRE-Luc, which was being transfected into fibroblasts. The application of RA flavonoids onto the transfected cells induced the transcriptional activity of HRE. To account for the transcriptional activation after the treatment of flavonoids, the expression of hypoxia-inducible factor-1α (HIF-1α) was markedly increased: The increase was in both mRNA and protein levels. In addition, the degradation of HIF-1α was reduced under the effect of flavonoids. The regulation of HIF-1α therefore could account for the activation of EPO expression mediated by the RA flavonoids. The current results therefore reveal the function of this herb in enhancing hematopoietic functions.

KEYWORDS: Flavonoid, Radix Astragali, hypoxia-inducible factor, erythropoietin

INTRODUCTION

Traditional Chinese medicines (TCMs), applied by Chinese medicinal practitioners for thousands of years in China, have played an important role in health maintenance and clinical therapy. The usage of TCM has attracted more and more attention for serving as complementary health food supplements, in addition to western medicines, with low toxicity and fewer or even no complications.[1,2] Radix Astragali (RA; Huangqi), the dried root of *Astragalus membranaceus* (Fisch.) Bunge or *A. membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) P. K. Hsiao, is one of the most widely used Chinese herbs, as a health food supplement, to reinforce "qi" (vital energy).[3] Its critical role in an herbal decoction Danggui Buxue Tang, a simple combination of RA and Radix Angelicae Sinensis (RAS) in a 5:1 ratio, has been demonstrated to improve health by raising the "qi" and nourishing the "blood" (body circulation).[4−6]

Pharmacological studies and clinical practice have demonstrated that RA possesses many biological functions, including hepatoprotective effects, neuroprotective effects against ischemic brain injury, hematopoietic, antioxidative, antihypertensive, immunological properties, cardiotonic, and antiaging activities, and can strengthen the superficial resistance, which means improving the immunity of humans.[7,8] Chemical investigations into RA resulted in the discovery of several kinds of bioactive components associated with effects on human health, that is, isoflavonoids, triterpene saponins, and polysaccharides.[8] In view of the chemical composition as well as their contents, the 3 year old RA collected from the Shanxi region in China was shown to have the best quality.[3,4]

A classic physiological response to hypoxia in human is the up-regulation of erythropoietin (EPO) in our blood circulation, which regulates the red blood cell production: EPO, a glycoprotein, is majorly produced by the kidney and liver.[9] Failure to increase the amount of circulating blood EPO under hypoxic stress could lead to anemia.[10] The key regulator of EPO expression is hypoxia-inducible factor (HIF), which is a transcription factor that modulates a wide range of processes, including erythrocytosis, angiogenesis, and cellular metabolism.[11] HIF, containing an inducible HIF-1α subunit and a constitutive HIF-1β subunit, is an oxygen sensor that plays a central role in the maintenance of oxygen homeostasis in body tissues.[12] Upon hypoxic stimulation, the activated HIF binds onto the hypoxia response element (HRE) located on the promoter region of the *EPO* gene. The binding of HIF with HRE subsequently triggers the transcription of the *EPO* gene. Under normoxic conditions, HIF-1α is proline hydroxylated, leading to a conformational change that promotes the binding to von Hippel Lindau protein (VLH) E3 ligase complex, which subsequently causes ubiquitination and proteasomal degradation.[13] Under hypoxic conditions, or

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in the presence of chemical hydroxylase inhibitors, HIF-1α degradation could be reduced and thus lead to its stabilization. As a result, the hypoxia, or inhibitor treatment, could enhance the expressions of HIF-1α and EPO.

RA is well-known to improve the hypoxia condition; however, the functional chemicals have never been revealed. Here, we revealed that the RA flavonoids including formononetin, ononin, calycosin, and calycosin-7-*O*-β-D-glucoside were able to induce EPO expression in cultured cells. Moreover, we provided different lines of evidence to support the notion that these flavonoids could induce EPO expression in kidney cells via two distinct mechanisms: (i) the transcriptional control that increases the amount of HIF-1α mRNA and (ii) the post-translational control that accumulates the HIF-1α protein via the reduction of HIF degradation in cultured kidney cells.

MATERIALS AND METHODS

Plant Materials and Chemicals. Three year old RA was collected from Shanxi province.[3] The authentication of plant materials was performed morphologically by one of the authors, Dr. Tina Dong, during the field collection. The corresponding voucher no. 02-10-4 for RA serves, as forms of the whole plant were deposited in Center for Chinese Medicine, HKUST. Formononetin, calycosin, and astragaloside IV were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and the purity was higher than 98.0%. Ononin, calycosin-7-*O*-β-D-glucoside, acetylastragaloside, and astragaloside II (purity >98%) were kindly provided by Prof. Pengfei Tu, Medical College of Peking University. These compounds were isolated previously from RA by repeated silica gel, Sephadex LH-20, and Rp-18 silica gel column chromatography: Their structures were elucidated by comparison of their spectral data (UV, IR, MS, ^{1}H NMR, and ^{13}C NMR) with those published references.[8]

Preparation of RA Extracts and Chemicals. The dry roots of RA (50 g) were extracted twice with distilled water (400 mL) at 100 °C for 2 h. The extract was centrifuged at 3000*g* for 10 min. The supernatant was freeze-dried (yield = 14.56 g) and kept at −20 °C. The dried extract was dissolved in phosphate-buffered saline and filtered through a 0.22 μm filter before use. The pure chemicals were dissolved by dimethyl sulfoxide (DMSO) before use.

Cell Culture. The human embryonic kidney (HEK) 293T fibroblast cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin in a humidified CO_2 (5%) incubator at 37 °C. All culture reagents were purchased from Invitrogen Technologies (Carlsbad, CA).

HEK293T has been shown to be an excellent in vitro model in studying the physiological regulation of EPO expression,[9] which is sensitive to hypoxia stress. To reveal the trophic effect of RA and its flavonoids in regulating EPO expression, different concentrations of RA extract and its flavonoids were applied onto the fibroblast cultures for 2 days.

Quantitative Real-Time Polymerase Chain Reaction (PCR). For the analyses of EPO, HIF-1α, and HIF-1β mRNA expressions in cultured HEK293T cells, total RNA was isolated by TRIzol reagent and reverse transcribed into cDNAs according to the manufacturer's instructions (Invitrogen). Real-time PCR was performed by using SYBR Green Master mix and ROX reference dye according to the manufacturer's instructions (Applied Bioscience, Foster City, CA). The primers were as follows: 5′-ACT TTC CGC AAA CTC TTC CG-3′ and 5′-TGA ATG CTT CCT GCT CTG GG-3′ for human EPO (330 bp; NM_000799.2); 5′-GCT TTA ACT TTG CTG GCC CCA GC-3′ and 5′-GCA GGG TCA GCA CTA CTT CGA AG-3′ for human HIF-1α (221 bp; NM_001530.3); 5′-ACT GGC AAC ACA TCC ACT GAT GGC-3′ and 5′-CTG AAG TGG AAA GCT GCT CAC G-3′ for human HIF-1β (253 bp; NM_001668.2). The 18S rRNA was used as an internal control in all cases, and its primer sequences were 5′-TGT GAT GCC CTT AGA TGT CC-3′ and 5′-GAT AGT CAA GTT CGA CCG TC-3′ (320 bp; NR_003286). SYBR green signal was detected by Mx3000ptm multiplex quantitative PCR machine (Stratagene, La Jolla, CA). Transcript levels were quantified by using ΔΔCt value method,[14] where the values of target genes were normalized by the 18S rRNA in the same sample at first before comparison. The PCR products were analyzed by gel electrophoresis and melting curve analysis to confirm the specific amplification.

DNA Construction and Transfection. The HRE (5′-TCG AGG CCC TAC GTG CTG TCT CAC ACA GCC TGT CTG ACG-3′) derived from the human *EPO* gene contains a highly conserved HIF-1 binding site (5′-TAC GTG-3′) and other unique cis-acting sequences (5′-CAC AG-3′) that are functionally essential for hypoxic induction.[6,15] Six HREs were synthesized, concatemerized, and then cloned in tandem (head-to-tail orientation) into pBI-GL vectors (BD Biosciences Clontech, San Jose, CA) that had a downstream reporter of firefly *luciferase* gene:[6] This vector was named as pHRE-Luc.[15] Cultured HEK293T cells were transiently transfected with pHRE-Luc by the calcium phosphate precipitation method.[16] The transfection efficient was over 80%, as determined by another control plasmid of having a β-galactosidase, under a cytomergalovirus enhancer promoter.

Luciferase Activity. After the drug treatment, luciferase assay was performed by a commercial kit (Tropix Inc., Bedford, MA). In brief, cultures were lysed by a buffer containing 100 mM potassium phosphate buffer (pH 7.8), 0.2% Triton X-100, and 1 mM dithiothreitol. The luminescent reaction was quantified in a Tropix TR717 microplate luminometer, and the activity was expressed as absorbance (up to 560 nm) per mg of protein. The luciferase activity was normalized by the fluorescent intensity of EGFP in the same amount of protein in each sample.

Western Blot and Antibodies. The stability of HIF-1α protein could be another crucial factor to account for the induction of HIF-1α protein, which involves the degradation pathway of HIF-1α. To reveal the degradation processes, two specific inhibitors for HIF-1α degradation were used. Desferrioxamine (DFO), a chemical hydroxylase inhibitor, inhibits the hydroxylation of HIF-1α, which results in a reduced HIF-1α degradation. Z-Leu-Leu-Leu-al (MG132), a cell-permeable proteasome inhibitor, reduces the degradation of ubiquitin-conjugated proteins including HIF-1α, which leads to the accumulation of HIF-1α and HIF-1α−OH. In cultured HEK293T, HIF-1α (an active form) and HIF-1α−OH (a degraded form) were recognized by Western blotting using specific antibodies.

Immunoblot analyses of HIF-1α, hydroxy-HIF-1α (HIF-1α−OH), and α-tubulin in cultured HEK239T cells, treated with different chemicals, were performed. The following primary antibodies were used for protein detection: monoclonal anti-HIF-1α from rabbit (Cell Signaling; 1:1000 dilution), monoclonal anti- HIF-1α−OH from rabbit (Cell Signaling; 1:1000 dilution), and monoclonal anti-α-tubulin from mouse (Sigma; 1:50000 dilution). The immune complexes were visualized using the enhanced chemiluminescence (ECL) method (Amersham Bioscience, Piscataway, NJ). The intensities of the bands in the control and different samples, run on the same gel and under strictly standardized ECL conditions, were compared on an image analyzer, using a calibration plot constructed from a parallel gel with serial dilutions of one of the samples.

Other Assays. Protein concentrations were measured routinely by Bradford's method (Hercules, CA). Statistical tests were done by using one-way analysis of variance. The significant differences between treatments were analyzed by independent *t* test of SPSS. Statistically significant changes were classed as *, where $p < 0.05$; **, where $p < 0.01$; and ***, where $p < 0.001$.

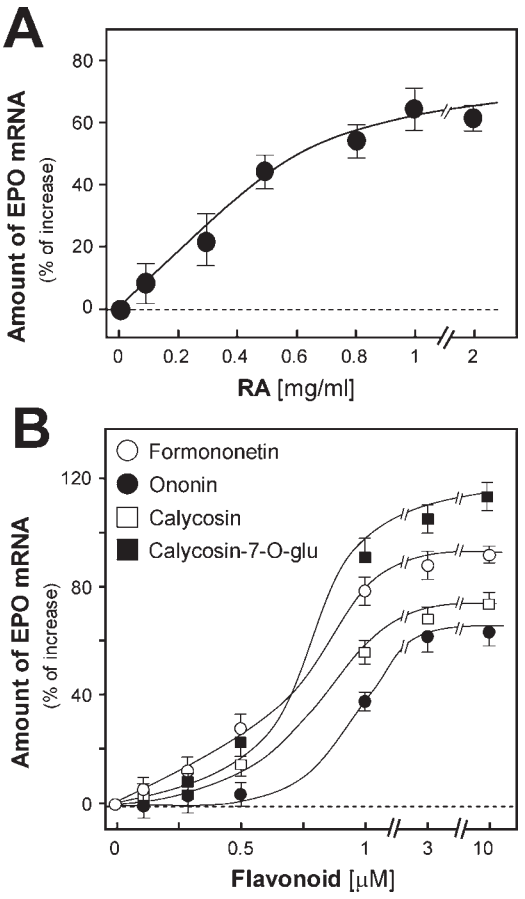

Figure 1. RA extract and flavonoids induce EPO expression in cultured HEK293T cells. (A) Cultured HEK293T cells were treated with 1 mg/mL of RA water extract for 48 h. Total RNAs were isolated from cultured HEK293T cells and then reversed transcribed (RT) into cDNAs for the detection of mRNAs encoding for EPO (330 bp) by real-time PCR analysis. The 18S rRNA (320 bp) served as an internal control. The PCR products were not shown. (B) Cultured HEK293T cells were treated with flavonoids at different concentrations for 48 h, and the amount of EPO mRNA was determined by real-time PCR (same as in A). Values are expressed as the percentage of increase to basal reading (untreated culture) and in mean ± SD, where $n = 4$, each with triplicate samples.

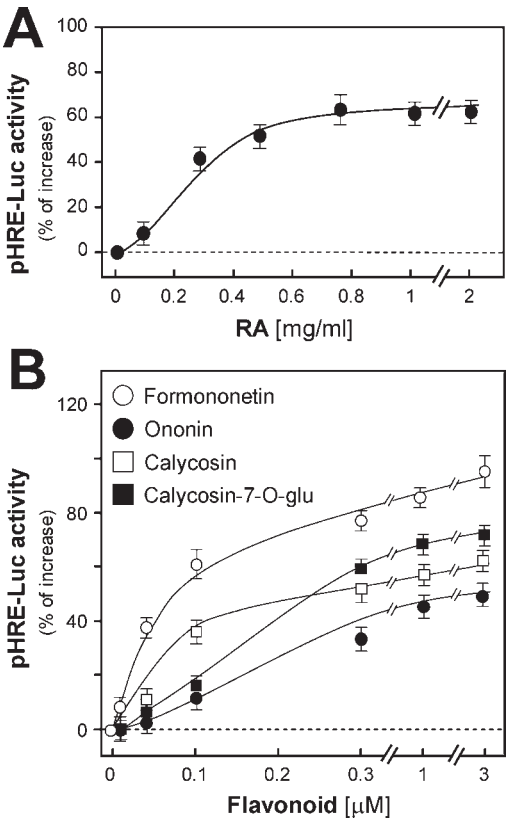

Figure 2. RA extract and flavonoids stimulate the HRE-mediated transcriptional activity in cultured HEK293T cells. (A) RA water extracts at different concentrations were applied onto pHRE-Luc-expressed fibroblasts for 48 h. The cell lysates were subjected to luciferase assay. (B) Different amounts of flavonoids at micromolar concentrations were tested in the pHRE-Luc-transfected cells. Values are expressed as the percentage of increase to basal reading (untreated culture) and in mean ± SD, where $n = 4$, each with triplicate samples.

RESULTS

RA Flavonoids Induce EPO Expression. The cytotoxicity of RA extract in the fibroblast cultures was tested. The results showed that 0.1−3 mg/mL RA extract did not change the cell morphology and the cell number, as revealed by microscopic examination and cell viability assay, respectively (data not shown), indicating the absence of cytotoxicity of the extract. Total RNA was collected from the treated cells and subjected to real-time quantitative PCR analysis by using specific primers flanking EPO mRNA. The RA-induced EPO expression was shown to be dose-dependent, with a peaked response at ∼1 mg/mL of RA extract, and the maximal induction was over 60% of increase (Figure 1A). Two classes of chemicals, flavonoid and astragaloside, are mainly found in RA extract.[3] In the regulation of EPO expression, the astragalosides did not induce the EPO expression: This negative effect was also applied to RA polysaccharide (Supporting Information table). However, RA flavonoids showed a robust induction of EPO mRNA. Four major RA flavonoids, including formononetin, ononin, calycosin, and calycosin-7-*O*-*β*-D-glucoside, were chosen for analysis. The application of these flavonoids in the cultured cells increased the level of EPO mRNA in a dose-dependent manner (Figure 1B). Calycosin-7-*O*-*β*-D-glucoside showed the highest EPO induction: The maximal induction was over ∼120% of increase (Figure 1B). In addition to the kidney fibroblast, we also tested the induction of flavonoids in cultured HepG2 liver cells, another cell line commonly used in the analysis of EPO expression. The results showed that pHRE-Luc activity had a high response to the RA flavonoids in HepG2 cells (Supporting Information, Figure 1).

To validate the response of the pHRE-Luc in transfected HEK293T cells, the cultures were exposed to hypoxia, serving as a positive control. The authentication of pHRE-Luc was confirmed by its activation in exposure to mineral oil layering, a method that was frequently used to mimic the effect of hypoxia.[17,18] The activation of pHRE-Luc activity by mineral oil layering, as a positive control, was in a time-dependent manner.[6]

The RA extract and RA flavonoids were applied onto pHRE-Luc-transfected fibroblasts. The application of RA extract increased the HRE-driven luciferase activity in a dose-dependent manner, and the maximal induction was over ∼60% of increase as compared to the background (Figure 2A). The four flavonoids (formononetin, ononin, calycosin, and calycosin-7-*O*-*β*-D-glucoside) could induce the transcriptional activity of HRE. The flavonoids-induced luciferase activity was in a dose-dependent manner, and

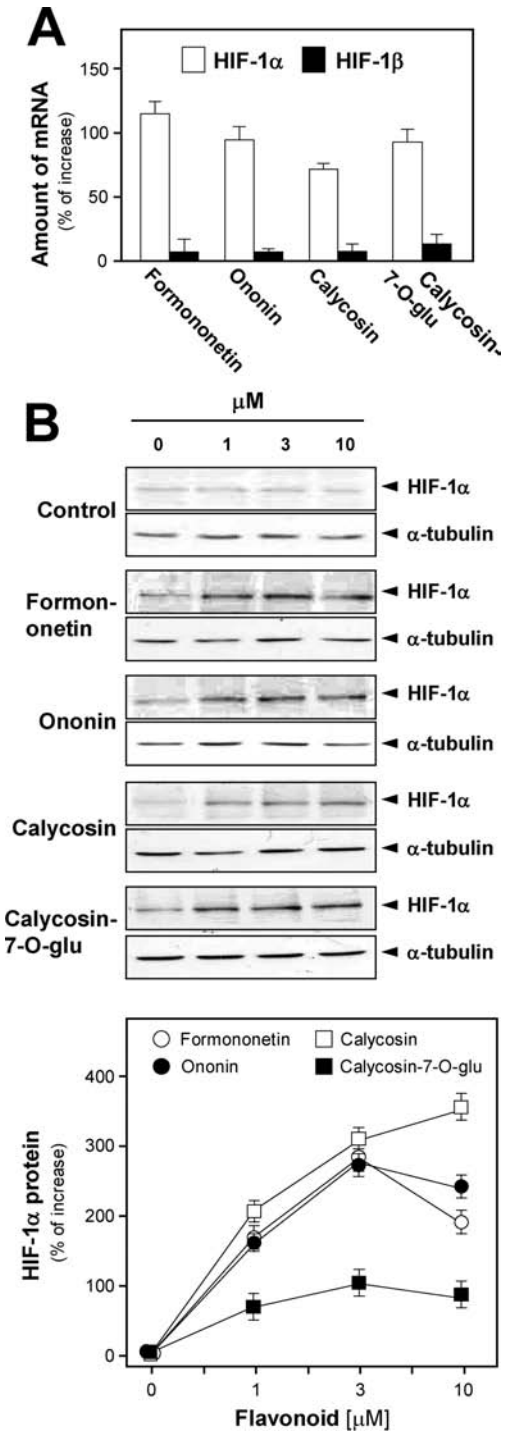

Figure 3. Flavonoids induce the accumulation of HIF-1α mRNA and protein. (A) Cultured HEK293T cells were treated with flavonoids at 1 μM for 6 h, and the amounts of HIF-1α (221 bp) and HIF-1β (253 bp) mRNAs were determined by real-time PCR. The PCR products were not shown. (B) Cultured HEK293T cells were changed with fresh medium for 3 h before the addition of flavonoids at different concentrations. The treatment was for 6 h. HIF-1α (~120 kDa) and α-tubulin (~55 kDa) were revealed by using specific antibodies (upper panel). The control is DMSO at 0.2%. The loading control was the amount of α-tubulin. The quantitation from the blots was shown by a densitometer (lower panel). Values are expressed as the percentage of increase to basal reading (untreated culture) and in mean ± SD, where n = 4, each with triplicate samples.

the peak of induction was at ~1 μM. The EC_{50} values of flavonoids were 0.05, 0.56, 0.66, and 1.47 μM for formononetin, ononin, calycosin, and calycosin-7-*O*-β-D-glucoside respectively. The most potent flavonoid in the HRE activation was formononetin: The EC_{50} was 0.05 μM that was over 5-fold higher than the others (Figure 2B).

Flavonoids Induces the HIF-1α mRNA and Protein. To account for the regulatory mechanisms of HIF in flavonoid-induced HRE activation, the HIF signaling cascade involved the accumulation of HIF-1 expression was analyzed. First of all, the amount of mRNAs encoding HIF-1α and HIF-1β was determined in flavonoid-treated fibroblasts. The level of HIF-1α mRNA was increased to ~100% by the application of flavonoids (Figure 3A): The induction was rather similar to the four tested flavonoids. However, the expression of HIF-1β mRNA did not significantly alter by the application of flavonoids. The level of HIF-1α protein was also determined here. Application of flavonoids onto cultured HEK293T cells induced the expression of HIF-1α protein: The induction was in a dose-dependent manner (Figure 3B). This stimulatory effect of flavonoids was from ~100 to 350% of the induction, and the maximal induction was achieved at 3 μM until to 10 μM (Figure 3B).

Flavonoids Increase the Translation of HIF-1α. The increase in protein translation could account for the induction of HIF-1α protein. Thus, phenethyl isothiocyanate (PEITC), a translation inhibitor of HIF,[19] was employed in our assay. The application of PEITC in the cultures fully blocked the flavonoid-induced HIF-1α protein expression (Figure 4A). In line to this observation, the levels of HRE-mediated luciferase activity and EPO mRNA were both significantly reduced by the application of PEITC (Figure 4B).

Flavonoids Reduce the Degradation of HIF-1α. Serving as a positive control, DFO robustly accumulated HIF-1α expression and reduced the amount of HIF-1α−OH (Figure 5A). In contrast, the application of MG132 in the cultures increased both forms of HIF-1α in a time-dependent manner (Figure 5A). The level of α-tubulin remained unchanged, serving as a loading control. In flavonoid-treated cultures, the amounts of HIF-1α and HIF-1α−OH were increased in a dose-dependent manner (Figure 5B). The responses of flavonoid treatment were, however, very similar to that of MG132 treatment, which suggested that flavonoids could block the proteasome-mediated degradation of HIF-1α, similar to that of MG132.

To further confirm this possibility, the cultures were treated with both DFO and MG132, and the expression patterns of HIF-1α and HIF-1α−OH were compared under different experimental conditions. When the cells were treated with DFO only, the inhibition of hydroxylation could lead to the increase of HIF-1α, as well as the decrease of HIF-1α−OH. On the other hand, the pretreatment of MG132 for 3 h, then cotreatment of MG132 and DFO for another 3 h, and the expression of HIF-1α increased; however, the expression of HIF-1α−OH did not decrease as a result of proteasome inhibition. Interestingly, under the same treatment of flavonoid with DFO, the expression patterns of HIF-1α−OH were highly similar to that of MG132 with DFO, that is, high expression of HIF-1α but no change on HIF-1α−OH (Figure 6A,B).

DISCUSSION

EPO is the hormone responsible for the production of red blood cell in the bone marrow. It is primarily produced by the

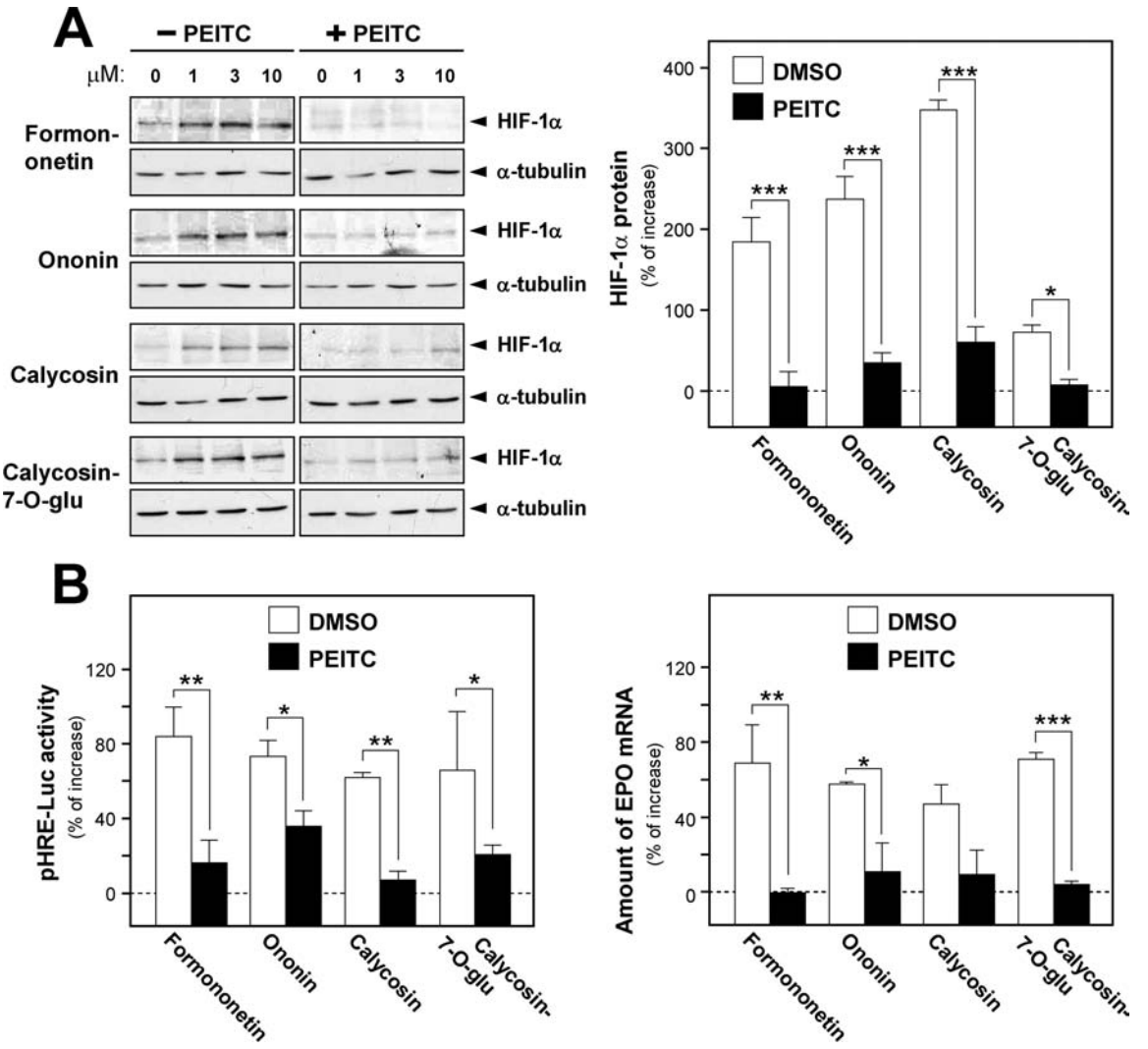

Figure 4. PEITC, an inhibitor of HIF translation, blocks the effects triggered by RA flavonoids. (A) Cultured HEK293T cells were pretreated with DMSO (control at 0.2%) or PEITC (10 μM dissolved in 0.2% DMSO) for 3 h before the treatment by flavonoids with different concentrations (1−10 μM) for another 6 h. HIF-1α (~120 kDa) and α-tubulin (~55 kDa) were revealed by using specific antibodies (right panel). The left panel shows the quantitation from the blots by a densitometer. (B) Cultured HEK293T cells were transfected with pHRE-Luc and then were pretreated with DMSO (control at 0.2%) or PEITC (10 μM) for 3 h before the application of flavonoids (1 μM) for another 48 h. Luciferase assay was performed to measure the HRE-driven luciferase activity (right panel), and real-time PCR was performed to measure EPO mRNA (left panel). Values are expressed as the percentage of increase to basal reading (control culture) and in mean ± SD, where $n = 4$, each with triplicate samples. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

kidney when a drop in blood oxygen level is perceived, though 10−15% of the total EPO is derived from the liver.[20] Here, kidney and liver cells were used to reveal the EPO induction of RA. The results showed that the pHRE-Luc activity had a high response to the RA flavonoids in cultured HEK293T and HepG2 cells; the induction was similar between kidney and liver cells (Figure 5B and Supporting Information, Figure 1). Because most of the EPO is produced by kidney, it should make sense that a kidney cell line, HEK293T, was chosen as a model in our study.

Flavonoids are considered to be the major active ingredients in RA. Here, we provided different lines of evidence to further strengthen this notion that RA-derived flavonoids including formononetin, ononin, calycosin, and calycosin-7-*O*-β-D-glucoside possessed the activity in inducing the expression of EPO in cultured fibroblasts deriving from either kidney or liver. The EPO induction was mediated by an up-regulation of HIF-1α protein after the flavonoid treatments. On the basis of our findings, RA flavonoids could affect the EPO expression in two distinct pathways (see the summary in Figure 7): (i) The transcript encoding HIF-1α was markedly induced by the flavonoids, and (ii) the RA flavonoids reduced the degradation of HIF-1α−OH, as a result which significantly increased the levels of HIF-1α and HIF-1α−OH. The final outcome was the up-regulation of HIF-1α protein, the increase of dimerization of HIF-1α and HIF-1β, and subsequently the HIF dimer, could induce the transcription of the *EPO* gene. In the two regulating mechanisms, RA flavonoids could significantly affect the degradation of HIF-1α in 6 h, which is a short-term effect comparing the transcription pathway. In this case, this degradation effect is assumed to play a significant role in the regulation of HIF-1α protein expression in cells treated with RA flavonoids.

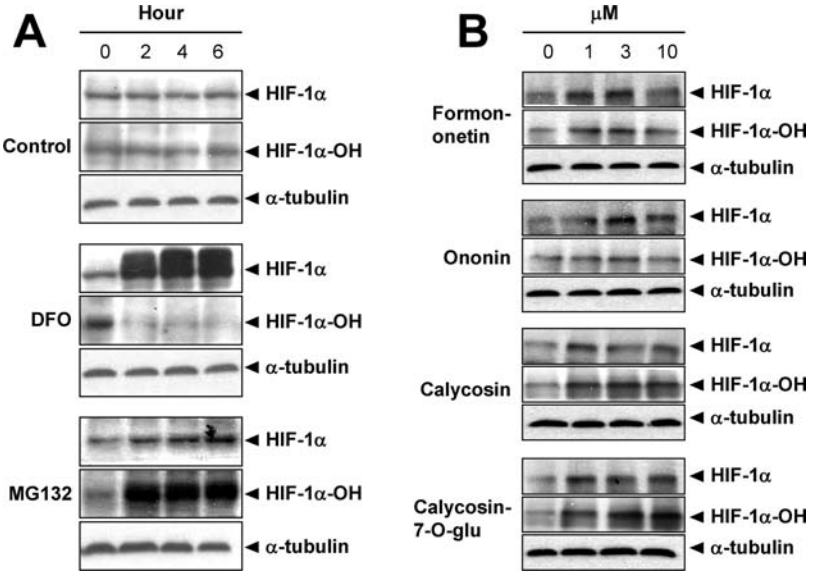

Figure 5. Flavonoids regulate the level of HIF-1α and HIF-1α−OH. (A) Cultured HEK293T cells were changed with fresh medium for 3 h before the addition of DFO (100 μM; dissolved in 0.2% DMSO) and MG132 (30 μM; dissolved in 0.2% DMSO) for 0−6 h. HIF-1α and HIF-1α−OH (both at ∼120 kDa) were revealed by specific antibodies by Western blot. α-Tubulin (∼55 kDa) served as a loading control. The control is DMSO at 0.2%. (B) Cultured HEK293T cells were treated with 0−10 μM flavonoids for 6 h. The expressions of HIF-1α, HIF-1α−OH, and α-tubulin were analyzed by specific antibodies as in panel A. The control is DMSO at 0.2%. Values are expressed as the percentage of increase to basal reading (untreated culture) and in mean ± SD, where n = 4, each with triplicate samples.

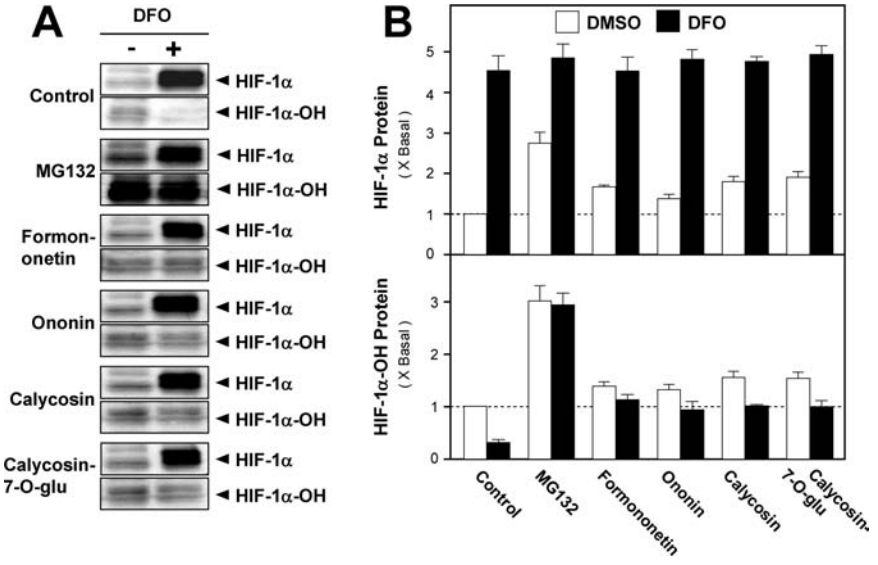

Figure 6. Cotreatment of flavonoids with DFO is similar to that of MG132. (A) Cultured HEK293T cells were pretreated with MG132 (30 μM) or flavonoids (all at 1 μM) for 3 h before the application of DFO (100 μM) together for another 3 h. The treatment of DFO for 6 h served as a positive control. HIF-1α and HIF-1α−OH (both at ∼120 kDa) were revealed by specific antibodies by Western blot. α-Tubulin (∼55 kDa) served as a loading control (not shown). (B) The quantitation from the blots was shown by a densitometer. Values are expressed as the fold of change as compared that to basal reading (untreated culture) and in mean ± SD, where n = 4, each with triplicate samples.

In general, the regulation of HIF depends mostly upon the level of HIF-1α mRNA. The level of HIF-1α mRNA could be up-regulated via endoplasmic reticulum stress during hypoxia or ischemia.[21] In our study, flavonoids could, indeed, induce the level of HIF-1α mRNA and HIF-1α protein. Could the increase in protein translation account for the induction of HIF-1α protein? Thus, PEITC, a translation inhibitor of HIF, was employed in our assay. The results suggested that flavonoids derived from RA possessed a direct effect in stimulating HIF-1α mRNA transcription and translation, which could partially account for the hematopoietic function of them in stimulating EPO expression as described here (Figure 4). On the other hand, the activation of HIF-1α has been shown to be modulated by growth factor-dependent signaling molecules such as Erk and phosphoinositide 3-kinase.[22,23] Among different regulators, the role of mitogen-activated protein kinase was no doubt to be essen-

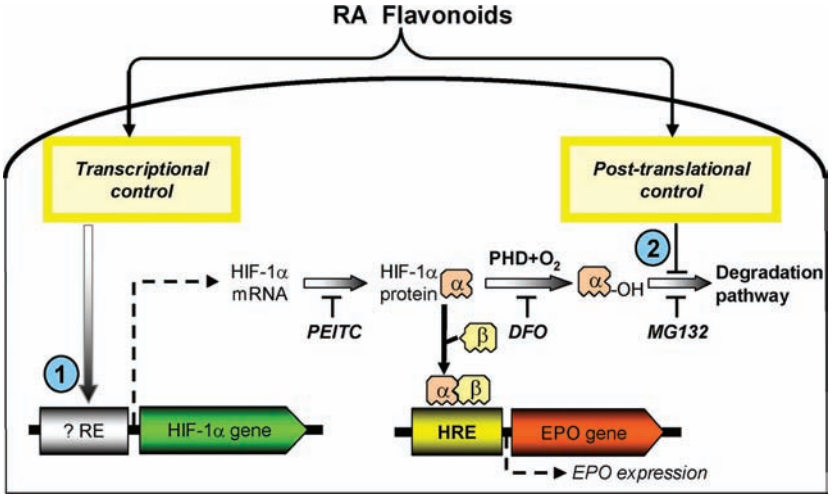

Figure 7. Possible mechanism of flavonoids derived from RA in directing the expression of EPO. The RA flavonoid-induced EPO expression is mediated by HIF-1α that triggers the transcription of EPO gene via HRE regulatory element. The regulation of HIF-1α by flavonoids could be at two levels: (1) transcriptional level in a result of an increase of the amount of HIF-1α mRNA and (2) post-translational level in a result of the reduction of HIF-1α degradation, mostly in the degradation of HIF-1α−OH. The final outcome therefore is the activation of EPO expression by the increased dimerization of HIF-1α and HIF-1β.

tial.[24,25] Previous studies supported the role of a Raf/MEK/ERK signaling in the regulation of HIF-1α activation and protein translation.[6,26] However, our results showed that none of the four RA flavonoids that we tested were able to induce the phosphorylation of Erk (Supporting Information, Figure 2).

In HIF signaling pathway, the degradation of HIF-1α protein plays major regulation of HIF-1α function.[27] HIF-1α is easily to be hydroxylated, and this hydroxylated form could be subjected to an ubiquitin-mediated degradation under normoxic conditions. Under hypoxia, the protein is stabilized and activated because of the reduced rate of degradation, that is, reduced level of HIF-1α−OH under hypoxia. Here, the RA flavonoids could not block the hydroxylation of HIF-1α but that affected the ubiquitination of HIF-1α: This was similar to the effect of MG132 (Figure 5). In line to our observation, flavonoids from different herbal sources have been demonstrated to induce the accumulation of HIF-1α by blocking the degradation pathway; for example, quercetin has been found to inhibit the ubiquitination of HIF-1/2α under normoxia.[28] Some flavonoids, including isoliquiritigenin, genistein and 7-hydroxyflavanone, exhibited inhibitory activities on human 20S proteasome.[29] As shown in here, RA flavonoids exhibited the properties of proteasome inhibitor, just like MG132 (Figure 5), which suggested that RA flavonoids could block the activities of human 20S proteasome. However, the mechanism needs to be explored further.

From our previous studies, we have shown that the application of Danggui Buxue Tang (a herbal decoction making of RA and RAS) could regulate EPO mRNA expression in cultured hepatocytes and renal cells.[5,6] RA is the major herb found in Danggui Buxue Tang, which therefore could account for the major function of this herbal decoction in triggering the expression of EPO. However, Danggui Buxue Tang has a more potent effect on EPO mRNA expression than RA.[6] We hypothesize that the boiling process of two herbs together in an optimal ratio (i.e., 5 to 1 of RA and RAS) could increase the solubility, or the stability, of the active ingredients in Danggui Buxue Tang. The final outcome is a higher amount of active ingredients in the decoction as compared to that of RA alone, RAS alone, or just RA + RAS (just mixing the extracts of RA and RAS together but boiled separately). On the other hand, the boiling of two herbs together was to eliminate the suppressive chemical, that is, ligustilide.[30] In addition, the RA-derived flavonoids including formononetin, ononin, calycosin, and calycosin-7-*O*-β-D-glucoside showed the promising activation on HRE-mediated luciferase activity in the transfected cells. The four tested flavonoids are the most abundant flavonoids within RA, as well as in Danggui Buxue Tang. Thus, the flavonoids within the decoction were responsible for the EPO activation. Actually, the chemical investigation into RA resulted in discovery of several bioactive components, that is, isoflavonoids, triterpene saponins, polysaccharides, γ-amino butyric acids, and various trace elements.[8] In the current findings, we reported four flavonoids that individually possessed different EPO-stimulating activities. In this case, the synergistic action among these effective flavonoids crucially accounts for the total effect of RA. Because of the chemical complexity, the percentage that the tested flavonoids could contribute to RA effects on HIF-1α has not been estimated.

■ ASSOCIATED CONTENT

Supporting Information. Table of the role of RA components in activating the HRE-mediated transcriptional activity in cultured HEK293T cells and figures of RA flavonoids that stimulate the HRE-mediated transcriptional activity in cultured HepG2 cells and RA flavonoids that do not induce the phosphorylation of Erk1/2. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

RA, Radix Astragali; TCM, traditional Chinese medicine; RAS, Radix Angelicae Sinensis; EPO, erythropoietin; HRE, hypoxia response element; HIF, hypoxia-inducible factor; PEITC, phenethyl isothiocyanate; DFO, desferrioxamine; MG132, Z-Leu-Leu-Leu-al.

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