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Salidroside stimulates the accumulation of HIF-1α protein resulted in the induction of EPO expression: A signaling via blocking the degradation pathway in kidney and liver cells

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ABSTRACT

Rhodiolae Crenulatae Radix et Rhizoma (Rhodiola), the root and rhizome of *Rhodiola crenulata* (Hook. f. et Thoms.) H. Ohba, has been used as a traditional Chinese medicine (TCM) to increase the body resistance to mountain sickness in preventing hypoxia; however, the functional ingredient responsible for this adaptogenic effect has not been revealed. Here, we have identified salidroside, a glycoside predominantly found in Rhodiola, is the chemical in providing such anti-hypoxia effect. Cultured human embryonic kidney fibroblast (HEK293T) and human hepatocellular carcinoma (HepG2) were used to reveal the mechanism of this hematopoietic function mediated by salidroside. The application of salidroside in cultures induced the expression of erythropoietin (EPO) mRNA from its transcription regulatory element hypoxia response element (HRE), located on *EPO* gene. The application of salidroside stimulated the accumulation of hypoxia-inducible factor-1α (HIF-1α) protein, but not HIF-2α protein: the salidroside-induced HIF-1α protein was via the reduction of HIF-1α degradation but not the mRNA induction. The increased HIF-1α could account for the activation of *EPO* gene. These results supported the notion that hematopoietic function of Rhodiola was triggered, at least partially, by salidroside.

1. Introduction

Rhodiola, the dry root of *Rhodiola crenulata* (Hook. f. et Thoms.) H. Ohba, has been used in traditional medical system. The therapeutic effects of this specialized herb include relieving the symptoms of short-term hypothyroidism, neuro-protection, cardio-protection, anti-fatigue, anti-depression and anti-tumor (Panossian et al., 2010; Zubeldia et al., 2010). In particular, Rhodiola in animals was shown to possess a potent adaptogenic activity in response to the exposure of multiple-stressed conditions (Gupta et al., 2008; Zheng et al., 2011). The adaptogenic activity of Rhodiola was found to be enriched by water extraction, and these water soluble chemicals functioned to improve the anabolic state (Gupta et al., 2009), i.e. increase the oxygen uptake in the body.

Salidroside, one of the major phenylpropanoid glycosides derived from Rhodiola, is the chemical marker to evaluate the quality of Rhodiola (Shao, 2010). The content of salidroside in Rhodiola could reach 0.50% of dry weight. Possible functions of salidroside have been proposed. Salidroside was shown to reduce the proliferation of cancer cells, to reduce aging processes and to protect neuron against stresses (Hu et al., 2010; Mao et al., 2010; Zhang et al., 2010). On the other hand, the erythropoiesis in TF-1 cells could be promoted by salidroside (Qian et al., 2011). In line to this hematopoietic function, the expression of erythropoietin (EPO) in cultured 293T kidney fibroblasts was markedly stimulated by the water extract of Rhodiola (Zheng et al., 2011). Therefore, we hypothesize that salidroside could account for the hematopoietic function of Rhodiola via inducing the expression of EPO.

EPO, produced from human kidneys and human livers, is a glycoprotein hormone, which promotes the formation of red blood cells in the bone marrow (Dame et al., 2006). By resisting the survival of erythroid progenitor cells, as well as inducing their differentiation and proliferation, EPO increases the production of blood cells (Sasaki et al., 2000). In addition, EPO prevents neuronal apoptosis after cerebral ischemia or brain injury, and which involves also in wound healing (Haroon et al., 2003; Sirén et al., 2001). The transcriptional rate of *EPO* gene is regulated by hypoxia-inducible factors (HIFs) comprised of HIF-α and HIF-β. Until now, three HIF-α subunits (HIF-1α, HIF-2α, HIF-3α) and three HIF-β subunits (HIF-1β/ARNT1, ARNT2 and ARNT3) are identified. However, the expression of EPO was shown to be dominantly regulated by HIF-2α (Ratcliffe, 2007). By using DNA-protein binding analysis, an isolated hypoxia response element (HRE) deriving from the EPO locus

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could bind HIF-1α preferentially, while the HRE in the EPO 3′ enhancer could bind HIF-2α preferentially (Rankin et al., 2007). In parallel, HIF-1α and HIF-2α appeared to contribute the expression of EPO in tissue specifically: the renal EPO transcription was induced equally by HIF-1α and HIF-2α, and the brain EPO was mainly induced by HIF-2α during hypoxia (Yeo et al., 2008). These evidences indicated that HIF-1α may also contribute to EPO regulation. In addition, HIF-1α has been proposed to play an important role in an oxygen-sensing (Wenger, 2002). The expression of HIF-α subunit is specifically regulated by oxygen environment, while HIF-β subunit is constitutive expressed (Benizri et al., 2008). Under normoxic conditions, HIF-α protein is proline hydroxylated by specific prolyl hydroxylases (PHD). Then, the von Hippel–Lindau tumor suppressor protein (pVHL) binds to a HIF-derived peptide, and subsequently HIF-α protein is destructed by ubiquitination and proteasomal degradation (Ivan et al., 2001). Under hypoxia condition, HIF-α protein is stabilized, and thereof which dimerizes with HIF-β protein and binds onto HRE located on *EPO* gene in triggering the transcription.

In order to reveal the mechanism of salidroside in hematopoiesis, cultured human embryonic kidney fibroblast (HEK293T) and human hepatocellular carcinoma (HepG2) were used in testing the activation HIF-1α. The result could account the adaptogenic activity of Rhodiola in response to hypoxia.

2. Materials and methods

2.1. Materials

Salidroside was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and the purity was higher than 98.0%.

2.2. Cell culture

Human embryonic kidney fibroblast HEK293T cells (CRL-1573) and human hepatocellular carcinoma HepG2 cells (HB-8065™) were 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).

2.3. Quantitative real-time PCR

For the analyses of EPO, HIF-1α and HIF-2α mRNAs in cultures, the cells were treated with various drugs. 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 PCR primers were: 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) (Gao et al., 2008); 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′-CTG TCT CTC CAC CCC ATG TCT C-3′ and 5′-GAA GAA CCT CAG GGG TGG GAG-3′ for human HIF-2α (232 bp; NM_001430.4); 5′-TGT GAT GCC CTT AGA TGT CC-3′ and 5′-GAT AGT CAA GTT CGA CCG TC-3′ for 18S rRNA (320 bp; NR_003286). The 18S rRNA was used as an internal control in all cases. The SYBR green signal was detected by Mx3000ptm multiplex quantitative PCR machine (Stratagene, La Jolla, CA). Transcript levels were quantified by using ΔΔCt value method, where the values of target genes were normalized by 18S rRNA in the same sample before the comparison. The PCR products were analyzed by gel electrophoresis, and the melting curve analysis was to confirm the specific amplification.

2.4. DNA construction and transfection

The HRE (5′-TCG AGG CCC TAC GTG CTG TCT CAC ACA GCC TGT CTG ACG-3′) derived from 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 (Zheng et al., 2010). Six HREs were synthesized, concatemerized and then cloned in tandem (head-to-tail orientation) into pBI-GL vectors (BD Biosciences Clontech, San Jose, CA) upstream of a reporter of firefly *luciferase* gene: this vector was named as pHRE-Luc. Cultured HEK293T cells, or HepG2 cells, were transiently transfected with pHRE-Luc by the calcium phosphate precipitation method (Zheng et al., 2010). The transfection efficiency was about 80%, as determined by another control plasmid of having a gene of β-galactosidase under a cytomergalovirus enhancer promoter.

2.5. Western blot and antibodies

Cultured HEK239T cells, or HepG2 cells, were treated with different drugs, and then which were collected in high salt lysis buffer (10 mM HEPES, pH7.5, 1 mM EDTA, 1 mM EGTA, 1 M NaCl and 0.5% Triton X-100) containing 10 μg/ml leupeptin, 10 μg/ml aprotinin and 5 mM benzamidine HCl. The lysate was added with gel electrophoresis buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, and 2% 2-mercaptoethanol), and which was denatured at 100 °C for 5 min. The lysate was subjected to 10% SDS-PAGE and western blotting. The expressions of HIF-1α, hydroxylated HIF-1α (HIF-1α-OH), EPO and α-tubulin (a loading control) were identified by Western blot analysis. The antibodies (in 1: 1,000 to 2,000) to HIF-1α, HIF-1α-OH and HIF-2α (1:1,000; Cell Signaling, Danvers, MA); α-tubulin (1:50,000; Sigma); HRP-conjugated anti-rabbit secondary (1:5,000) and anti-mouse secondary (1:10,000; Zymed Laboratories, San Francisco, CA) were employed here. The immune complexes were visualized using the enhanced chemiluminescence (ECL) method (Amersham Biosciences). 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.

2.6. Other assays

The luciferase assay was performed by a commercial kit (Tropix, 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. The protein concentrations were measured routinely by Bradford's method (Hercules, CA). Statistical tests were done by using one-way ANOVA. Statistically significant changes were classed as [*] where $P<0.05$; [**] where $P<0.01$ and; [***] where $P<0.001$.

3. Results

3.1. Salidroside induced EPO mRNA expression in cultured cells

To investigate the effect of salidroside on EPO mRNA expression, salidroside was applied onto cultured HEK293T and HepG2 cells for 24 h. This salidroside treatment did not change the cell morphology and the cell number, as revealed by microscopic examination and cell viability assay, respectively (data not shown). The expression of EPO mRNA was analyzed by real-time quantitative PCR by using specific primers. The EPO mRNA expression was induced in a dose-dependent manner in both cell lines. In cultured HEK293T cells, the maximal induction was