Inhibitory effects of 17β-estradiol or a resveratrol dimer on hypoxia-inducible factor-1α in genioglossus myoblasts: Involvement of ERα and its downstream p38 MAPK pathways

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Abstract. Deficiency in the functioning of the genioglossus, which is one of the upper airway dilator muscles, is an important cause of obstructive sleep apnea/hypopnea syndrome (OSAHS). Estrogens have been reported to inhibit hypoxia-inducible factor-1α (HIF-1α) expression in hypoxia, regulating its target genes and exerting protective effects on the genioglossus in chronic intermittent hypoxia (CIH). This study aimed to investigate the role of 17β-estradiol (E_2) and a resveratrol dimer (RD) on HIF-1α and the underlying mechanism. Mouse genioglossus myoblasts were isolated and cultured, and the estrogen receptor α (ERα) shRNA lentivirus was used for gene knockdown. Then MTT assay was used to determine the effects of E_2 and RD on the viability of the cells. Cells in different groups were treated with different agents (E_2, or RD, or E_2 and SB203580), incubated under normoxia or hypoxia for 24 h, and then expression levels of HIF-1α, ERα, ERβ, total-p38 MAPK and phospho-p38 MAPK were detected. We observed that both E_2 and RD inhibited the overexpression of HIF-1α induced by hypoxia at the mRNA and protein levels, and these effects were eliminated by genetic silencing of ERα by RNAi. In addition, we found that E_2 activated p38 MAPK pathways to inhibit HIF-1α expression. On the whole, ERα may be responsible for downregulation of HIF-1α by E_2 or RD via activation of downstream p38 MAPK pathways.

Introduction

Obstructive sleep apnea/hypopnea syndrome (OSAHS) is a common disorder characterized by repetitive narrowing or collapse of the upper airway (UA) during sleep, resulting in a recurrent reduction or cessation of airflow. As periods of apnea/hypopnea occur intermittently, OSAHS is associated with chronic intermittent hypoxia (CIH). It is a multifactorial syndrome, which is mainly caused by narrow anatomic structure and defective functioning of UA (1). However, the pathophysiology of OSAHS remains incompletely understood. Although most patients with OSAHS have a narrow UA, for some individuals who have an anatomical predisposition to UA collapse, their UAs may still be maintained opened by UA dilator muscles. The state of UA depends on the balance between positive intraluminal pressure to open the airway and negative surface tension to keep it closed (2). There is substantial evidence supporting that UA dilator muscles play important roles in maintaining airway patency (3). Exposure to CIH, a result of repetitive narrowing or collapse of UA, promotes the activation of UA dilator muscles, causes a transition from slow to fast in muscle fiber types (4), and reduces the endurance of dilator muscles (5). Genioglossus, an important UA dilator muscle, is the main tongue muscle which exerts forward propulsion to the tongue and contracts in coordination before the diaphragm contracts. As for the important role of the genioglossus in maintaining UA patency, it is referred to as the safeguard of the UA (6).

Several studies have suggested that intermittent hypoxia promotes the expression of hypoxia-inducible factor-1 (HIF-1) and activates serial reactions to hypoxia (7-10). HIF-1 is a heterodimeric transcription factor composed of a hypoxia-inducible HIF-1α subunit and a consistently expressed HIF-1β subunit, which is well known for regulating a wide range of genes in response to hypoxia. The activation of HIF-1 depends on HIF-1α, which is normally kept low in cells by proteosomal degradation but is stabilized and transferred into the nucleus under hypoxia. HIF-1α was found to exhibit higher expression in skeletal muscles than other non-muscle tissues in normoxic conditions, indicating that it plays an important role in skeletal muscles (11). In our previous study, HIF-1α was verified to have regulating ability in genioglossus myogenesis in hypoxia (12). We also found that 17β-estradiol (E_2) exerts protective effects of fatigue resistance on the genioglossus in CIH rats (13,14), and these effects were coincident with the downregulation of

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HIF-1α (12,13). HIF-1α was found to be destabilized by estrogen treatment even under hypoxic conditions. This raises the question of how estrogen downregulates the expression of HIF-1α.

The genomic effect of estrogen is mediated by two estrogen receptor (ER) isoforms, ERα and ERβ, which belong to the family of nuclear receptors. The varying tissue-specific distribution and expression level of ERα and ERβ is the basis of the different biological effects of estrogen (15,16). A series of investigators found clear expression of ERα but weak or undetectable expression of ERβ in skeletal muscle in mice (17,18). It can be inferred that ERα may be of great significance in skeletal muscle.

To date, expanding the upper airway is the main method in the clinical treatment and management of OSAHS, including continuous positive airway pressure (CPAP), oral appliance and surgical treatment. However, each of these methods is associated with many defects and complications. Some researchers have discovered the possibility of medical therapy for OSAHS in recent years (19), but there are still no effective pharmacotherapies for individuals with OSAHS. Several researchers have indicated that female hormones may possess protective effects against OSAHS (20,21). A previous study confirmed that estrogen may protect the function of the upper airway (22). However, estrogen cannot be used for patients with OSAHS due to its side effects such as coronary heart disease, stroke and breast cancer. To limit the side effects of estrogen, estrogenic compounds are needed. Phytoestrogens, due to their similar structure with estrogens, have estrogenic effects via ERs and exhibit fewer side effects and long-term health benefits (prevention of osteoporosis, cardiovascular disease and breast cancer) (23). Resveratrol (Fig. 1A), a polyphenol found in a variety of plants, is such a type of phytoestrogen. Recently, Zhong *et al* reported a new approach with which to synthesize derivatives of resveratrol, which have similar pharmacological activities as resveratrol. These derivatives have increased availability than resveratrol and may be alternatives to estrogen in various therapies (24). One type of resveratrol dimer (RD), an endo-shifted olefin isomer of parthenocissin A (Fig. 1B), is testified to have considerable estrogenic properties and minimum cytoxia in pre-experiments. Studying the effects of RD on HIF-1α and comparing the results with those obtained from E_2 may be the first move to explore the possibility of RD replacing E_2 in the medical therapy for OSAHS.

In the present study, we isolated genioglossus myoblasts and silenced ERα to investigate the effect of E_2 and RD on HIF-1α and the underlying mechanism. Our study may aid to elucidate the molecular mechanism of E_2 and RD involved in the effects on the physical properties of the genioglossus and contribute to our future study of medical therapy for OSAHS.

Materials and methods

Materials. 17β-estradiol, SB203580 and MTT solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Resveratrol dimer was a kind gift of Professor X. Sun and coworkers (School of Pharmacy, Fudan University, Shanghai, China). The bicinchoninic acid (BCA) protein assay kit was obtained from Beyotime Institute of Biotechnology (Jiangsu, China). TRIzol reagent, expression vector kit with GFP and packaging mix were obtained from Invitrogen (Carlsbad, CA, USA). Lipofectamine 2000, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY, USA). Monoclonal anti-HIF-1α antibody, anti-ERα antibody and anti-ERβ antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-p38 antibody and anti-p-p38 antibody were purchased from Cell Signaling Technology (Boston, MA, USA). IRDye800-conjugated secondary antibodies were procured from Rockland Immunochemicals (Gilbertsville, PA, USA).

Primary cultivation and identification of genioglossus myoblasts. Genioglossus myoblasts were isolated and cultured as previously described (14). All procedures were approved by the Animal Care Committee of Tongji University. Under sterile conditions, the genioglossus of 2- to 3-day-old C57BL/6J mice was excised and minced with surgical scissors and forceps. After being transferred to a 15-ml centrifuge tube, the muscle slurry was enzymatically digested with 0.05% type II collagenase at 37˚C for 40 min, and centrifuged at 200 x g for 1 min. Further digestion was initiated in 0.25% trypsin-EDTA at 37˚C for 30 min, and stopped by the addition of 20% FBS (HyClone, Logan, UT, USA). Then, a 75-μm sieve (Millipore, Billerica, MA, USA) was used to filtrate the dissociated cells, which was followed by 200 x g centrifugation for 1 min. The sediment was resuspended in DMEM supplemented with 25% FBS. Cells were plated on culture dishes after twice repeated differential attachment treatment. After reaching 80% confluence, the growth medium was replaced with normal medium (10% FBS in DMEM).

To assess whether the putative myoblasts have the capacity of differentiation, the culture medium was replaced with differentiation medium (2% horse serum in DMEM) when cell fusion reached 80%. The cells were observed and photographed 4 days after the induction of differentiation to evaluate their morphological appearance.

After the second passage, the putative myoblasts were seeded onto 6-well plates at a density of $2x10^4$ cells/ml. After reaching 60% confluence, plated cells were fixed with paraformaldehyde for 15 min, then blocked with 5% bovine serum albumin [BSA, dissolved in phosphate-buffered saline (PBS)] for 1 h at room temperature, and incubated with 1:500 α-sarcomeric actin monoclonal antibody overnight at 4˚C, and then HRP-conjugated goat anti-mouse secondary antibody for 1 h. After being washed with PBS, the cells were incubated using the SABC kit for 30 min and then stained using a diaminobenzidine (DAB; Beyotime Institute of Biotechnology) kit according to the manufacturer's instructions. Fibroblasts were treated in the same way as the negative control.

Lentivirus production, ERα gene silencing and selection by flow cytometry. The ERα-knockdown shRNA was constructed by inserting the ERα shRNA fragment into empty plasmid pLKO.1. The DNA fragment for ERα was obtained with oligonucleotide forward, 5'-GGAGAATGTTGAAGCACAAGC-3' and reverse, 5'-GCTTGTGCTTCAACATTCTCC-3' sequences. The scrambled fragment was inserted as the control: ERα scrambled (ERα-NS) forward, 5'-GTTCTCCGA ACGTGTCACG-3' and reverse, 5'-ACGTGACACGTTCG GAGAAC-3'. 293T cells (obtained from the Type Culture

Table I. Nucleotide sequences of primers used for PCR amplification.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
ERα	AGGCGGCATACGGAAAGAC	CATTTCGGCCTTCCAAGTCA
HIF-1α	GACAATAGCTTCGCAGAATGC	TCGTAACTGGTCAGCTGTGG
β-actin	CCTCATGAAGATCCTGACCG	TGCCAATAGTGATGACCTGG

ERα, estrogen receptor α; HIF-1α, hypoxia-inducible factor-1α.

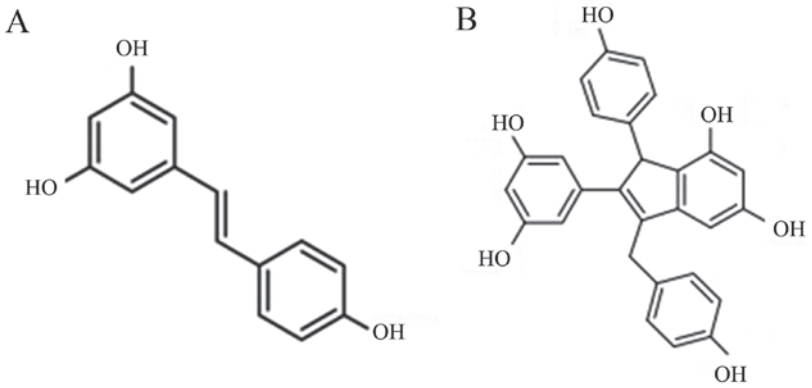


Figure 1. Chemical structures of resveratrol and its derivative used in this study. (A) Resveratrol. (B) Resveratrol dimer: endo-shifted olefin isomer of parthenocissin A.

Collection of the Chinese Academy of Sciences, Shanghai, China) were seeded onto 6-well plates at a density of $9x10^4$ cells/ml. After reaching 80% confluence, 293T cells were transfected with a mixture containing 2 μg pLKO.1 shRNA vector, packing plasmids (2 μg psPAX2 and 2 μg pMD2.G), 12 μl transfection agent Lipofectamine™ 2000 (Invitrogen) and 600 μl DMEM. After 48 h, the supernatant was harvested and filtered through 0.45-μm filters and the virus supernatants were obtained and then stored at 20˚C. For ERα gene silencing, the genioglossus myoblasts were plated onto 6-well plates and medium was replaced with virus supernatant 12 h afterward. Twenty-four hours later, the virus supernatant was removed and fresh medium was added. After reaching 80% confluence, transfected myoblasts (ERα-KD and ERα-NS) were digested with 0.25% trypsin-EDTA at 37˚C for 1.5 min, centrifuged at 200 x g for 5 min and resuspended in Hank's solution. Then, a 40-μm sieve was used to filtrate the cells, followed by 200 x g centrifugation for 5 min. The supernatant was discarded and the cells were resuspended in DMEM, and then sorted by flow cytometry. GFP-positive myoblasts were obtained and used for further experiments. The effectiveness of ERα gene silencing was verified by mRNA and protein expression.

Cell treatment and hypoxic conditions. The myoblasts, ERα-knockdown myoblasts (KD group) and ERα-scrambled myoblasts (NS group) were cultured at an atmospheric oxygen concentration (21% O_2, 5% CO_2; balance N_2) in an incubator. Myoblasts exposed to hypoxia were cultured in a hypoxia chamber (1% O_2, 5% CO_2; balance N_2). Cells in different groups were treated with vehicle-dimethyl sulfoxide (DMSO), or 1 μmol/l E_2, or 1 μmol/l RD, or 1 μmol/l E_2 and 10 μmol/l SB203580 (according to MTT assay and preliminary experiments), and then incubated under normoxia or hypoxia for 24 h.

MTT-based cytotoxicity assay of E_2 and RD. The effects of E_2 and RD on the myoblasts in normoxia or hypoxia were measured by MTT assay. Third passage myoblasts were seeded onto 96-well plates at $2x10^4$ cells/ml. Various concentrations of E_2 and RD (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/l) were added and the cells were incubated in a normoxic or hypoxic condition after reaching 40% confluence. The vehicles of the same concentration were added into fresh culture medium as control. After 24, 48 and 72 h, the plated cells were incubated with MTT solution (5 mg/ml) at 37˚C for 4 h, and then DMSO (100 μl/well) (both from Sigma-Aldrich) to dissolve the formazan precipitate. After been mixed for 30 min, viable cells were detected by measuring the absorbance at 595 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The mean optical density of 6 wells in the same group was used to assess the viability of the cells.

RNA isolation, RT-PCR, and real time PCR. RT-qPCR was employed to detect the mRNA expression of ERα and HIF-1α. Total RNA was isolated from the cells using TRIzol reagent, and then reverse transcribed to cDNA using PrimeScript RT reagent kit (Takara, Shiga, Japan) in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Isolated RNA and cDNA were both quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Real-time PCR was performed using SYBR Green as detection reagent on a 7500 real-time PCR system (7500; Applied Biosystems). The 20 μl PCR mixture contained 2 μl cDNA product, 10 μl SYBR Premix Ex Taq,

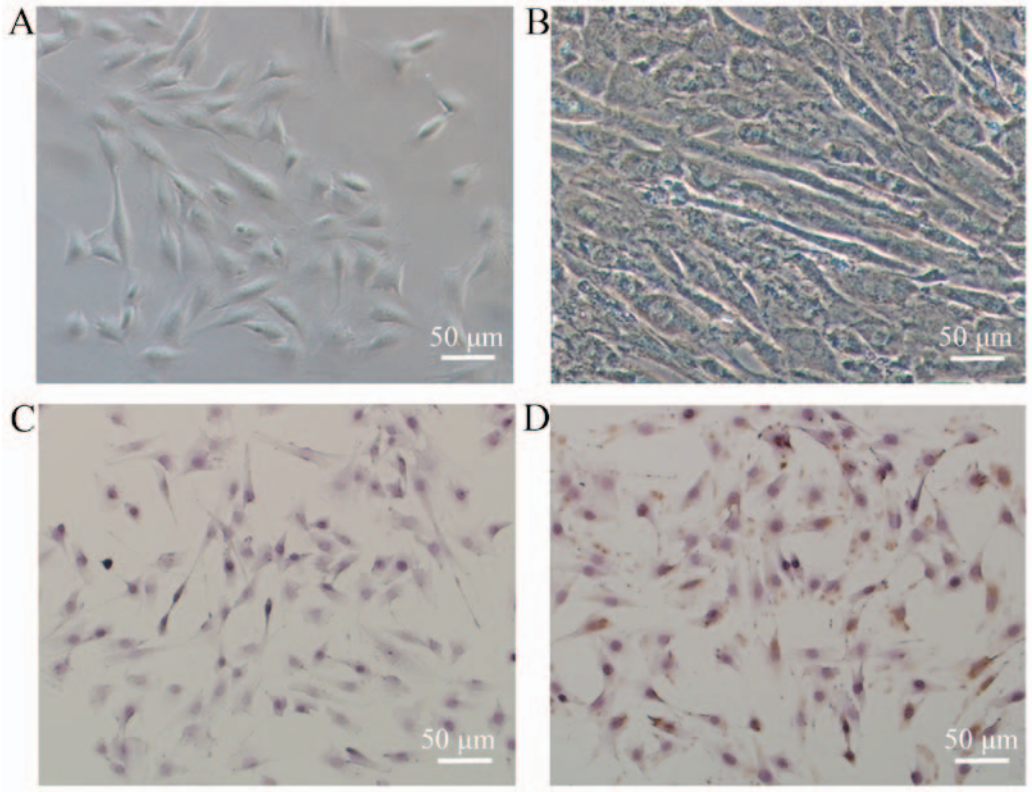


Figure 2. Characterization of mouse genioglossus myoblasts. (A) Genioglossus myoblasts showed a spindle form. (B) Myotubes derived from genioglossus myoblasts. (C) Fibroblasts demonstrated negativity for α-sarcomeric actin in immunocytochemical staining. (D) Immunocytochemical staining of genioglossus myoblasts revealed expression of α-sarcomeric actin.

0.4 μl ROX Reference Dye II, 6.8 μl RNase-free water, and 0.4 μl each of the forward and reverse primers. Gene-specific primers for ERα and HIF-1α are described in Table I. The first step of the PCR protocol was 95˚C for 30 sec, followed by 45 cycles of 95˚C for 5 sec, and 60˚C for 34 sec as the second step. A melting curve analysis was performed to ensure specificity of the PCR products. β-actin was used as a control, and the relative expression of the target genes was evaluated by a comparative CT method and normalized to the control. The average values were obtained from five repeated experiments.

Western blot analysis. Cell lysates were obtained using ice-cold RIPA buffer (Pierce, Rockford, IL, USA) with phenylmethanesulfonyl fluoride (1 mmol/l; Beyotime, Shanghai, China). Protein was quantified using the BCA protein assay. An equal amount of protein was denatured with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 95˚C for 5 min, separated on SDS-PAGE gels using a Bio-Rad apparatus, and then transferred to PVDF membranes (Millipore). After blocking with 5% bovine serum albumin (dissolved in TBST), the membranes were incubated with anti-HIF-1α antibody (1:500), anti-ERα antibody (1:1,000), anti-ERβ antibody (1:500), anti-p38 MAPK antibody (1:1,000), anti-p-p38 MAPK antibody (1:1,000), anti-β-actin antibody (1:2,500) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:2,500) at 4˚C overnight. After 3x10 min washes in TBST, the membranes were incubated with IRDye800-conjugated secondary antibodies (1:10,000) away from light for 20 min at room temperature. The immunoblots were imaged using the LI-COR Odyssey Infrared Imaging system (Li-COR, Lincoln, NE, USA). Anti-β-actin or anti-GAPDH was used as the loading control in the western blot analyses.

Data analysis. All quantitative results were obtained from triplicate samples. Data are expressed as mean ± standard deviation (SD). The independent samples t-test was used for the cell proliferation assay. One-way ANOVA was used to assess the difference between the control and experimental groups. A P-value <0.05 was considered to indicate a statistically significant result. The statistical program used was SPSS version 17.0 (SPSS Inc. Chicago, IL, USA).

Results

Characterization of mouse genioglossus myoblasts. The newly separated cells were spherical and displayed strong refractivity. Once the cells adhered, they became grossly fibroblast-like in shape and had a spindle form (Fig. 2A). After being induced with 2% horse serum, the genioglossus myoblasts were found to fuse into myotubes (Fig. 2B). More than 95% of isolated cells demonstrated positive immunostaining for α-sarcomeric actin, as assessed by immunocytochemical staining (Fig. 2D), while the fibroblasts (control) exhibited negativity (Fig. 2C).

The effects of E_2 and RD on genioglossus myoblast viability were determined by MTT assay (Fig. 3). The exposure of myoblasts to 10 μM E_2 resulted in a significant decrease in cell viability under both normoxic and hypoxic conditions, while at other lower concentrations E_2 had no inhibitory effect within 72 h. The same trend was found in

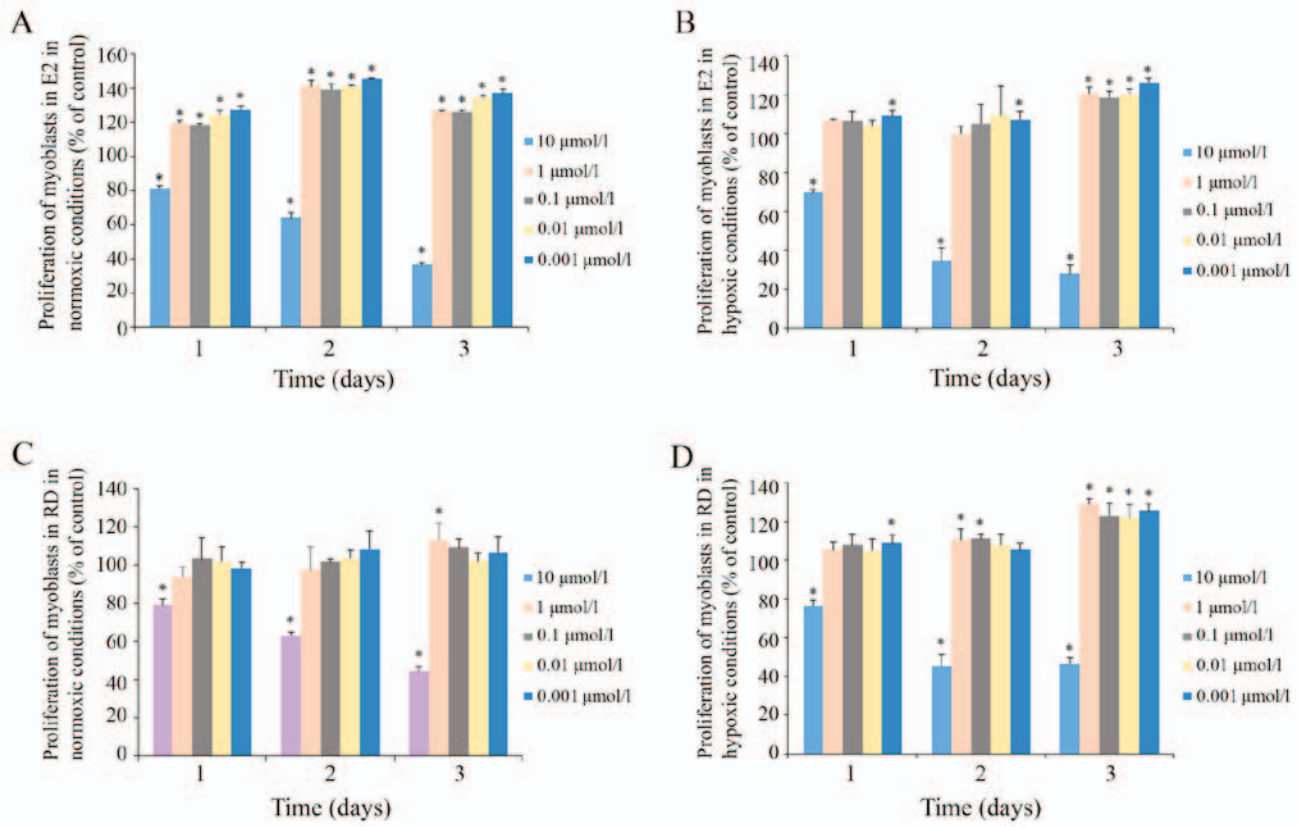


Figure 3. Effects of 17β-estradiol (E_2) and a resveratrol dimer (RD) on genioglossus myoblast proliferation (% of control). (A) Myoblast proliferation in E_2 in normoxic conditions. (B) Myoblast proliferation in E_2 in hypoxic conditions. (C) Myoblast proliferation in RD in normoxic conditions. (D) Myoblast proliferation in RD in hypoxic conditions. *P<0.05 compared with the control group.

myoblasts treated with RD. In addition, E_2 at 1, 0.1, 0.01 and 0.001 μM significantly improved the proliferation under a normoxic condition within 72 h. Therefore, concentrations of 1 μM were used for further experiments.

Role of ERα in the effects of E_2 and RD on the expression of HIF-1α

HIF-1α expression at the mRNA level. HIF-1α mRNA expression in the genioglossus myoblasts in hypoxia was significantly higher than that in normoxia (P<0.05). After the myoblasts were treated with E_2, HIF-1α mRNA was significantly lower than in mere hypoxia (P<0.05) but still higher than in normoxia (P<0.05). It was noteworthy that no significant difference was observed in HIF-1α mRNA between the RD group and E_2 group (P>0.05) (Fig. 4A).

To determine the role of ERα on HIF-1α expression in myoblasts, HIF-1α mRNAs were analyzed after ERα knockdown (KD group). The silencing efficiency of ERα was validated by RT-qPCR (Fig. 4C) and western blot analysis (Fig. 4D). As shown in Fig. 4B, hypoxia induced HIF-1α mRNA expression in myoblasts in the KD group (P<0.05) as in the NS group, while the inhibitory effects of E_2 and RD on hypoxia induced-HIF-1α were blocked by ERα knockdown, which implies that the inhibitory effect of E_2 and RD on HIF-1α is mediated by ERα.

HIF-1α expression at the protein level. The western blot results of HIF-1α expression were generally in accordance with those of RT-qPCR. In consideration of the possible effects of E_2 or RD on the expression of ERs, ERα and ERβ protein expression was detected as control. ERα expression in myoblasts in normoxia and hypoxia was at a similar level (P>0.05), but both E_2 and RD induced ERα expression notably (Fig. 5A). Weak expression of ERα was detected in the KD group (Fig. 5B). No significant difference in ERβ expression was observed in both the NS group and KD group (Fig. 5). As for HIF-1α expression, hypoxia induced the HIF-1α protein expression of myoblasts both in the NS group (P<0.05) and KD group (P<0.05). As shown in Fig. 5A, the HIF-1α protein level was significantly lower in the myoblasts treated with E_2 (P<0.05) or RD (P<0.05) than that in mere hypoxia. After silencing of ERα, however, the level was higher in E_2- or RD-treated myoblasts than under hypoxia (P<0.05) (Fig. 5B).

Taken together, these results indicated that RD, as well as E_2, inhibited the overexpression of HIF-1α in hypoxia; ERα knockdown prevented E_2- or RD-dependent HIF-1α suppression.

Role of p38 MAPK pathways in the effects of E_2 on the expression of HIF-1α. Several studies have demonstrated that estrogen can activate the MAPK pathway in a variety of cell types (25-27). Since the MAPK pathways are widely linked to the activation of HIF-1α, we aimed to ascertain whether these pathways are essential in the inhibitory effect of estrogen on HIF-1α in genioglossus myoblasts.

E_2 activates p38 MAPK pathways in hypoxia via ERα. Phosphorylated p38 MAPK (p-p38) (Fig. 6A) expression was

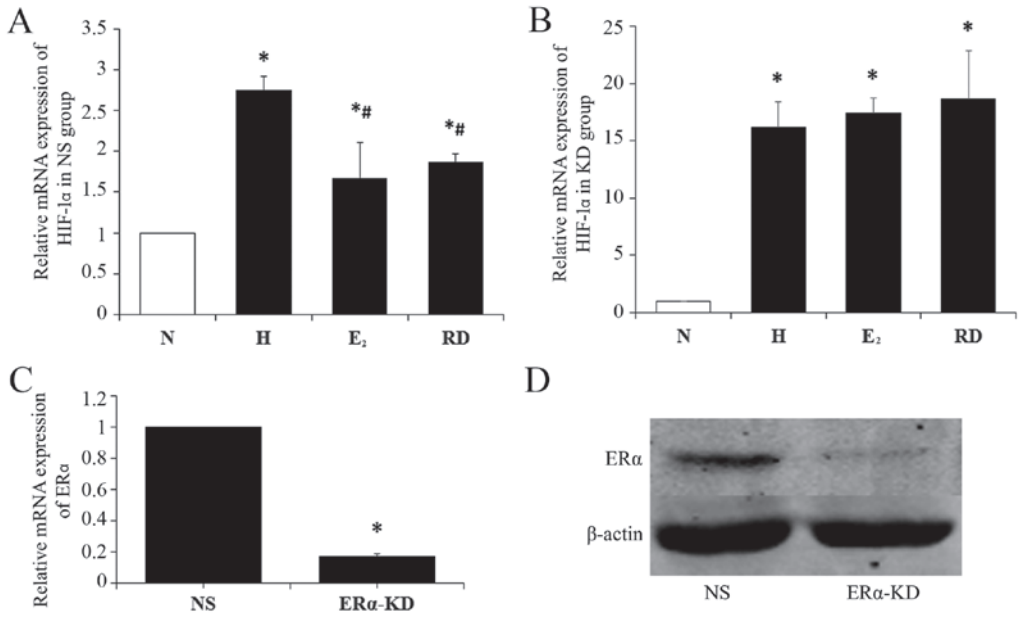


Figure 4. Comparison of hypoxia-inducible factor-1α (HIF-1α) mRNA expression in genioglossus myoblasts, relative to β-actin. (A) HIF-1α mRNA expression of myoblasts in the NS group. (B) HIF-1α mRNA expression of myoblasts in the KD group. (C) Estrogen receptor α (ERα) mRNA expression in genioglossus myoblasts, relative to β-actin, *P<0.05 vs. the NS group. (D) ERα protein expression in genioglossus myoblasts. NS, myoblasts transfected with negative scramble siRNA; KD, myoblasts transfected with ERα-siRNA. N, incubated in normoxia for 24 h; H, incubated in hypoxia for 24 h; E_2, incubated with 1 μmol/l 17β-estradiol in hypoxia for 24 h; RD, incubated with 1 μmol/l resveratrol dimer in hypoxia for 24 h. *P<0.05 vs. normoxia (N); #P<0.05 vs. hypoxia (H).

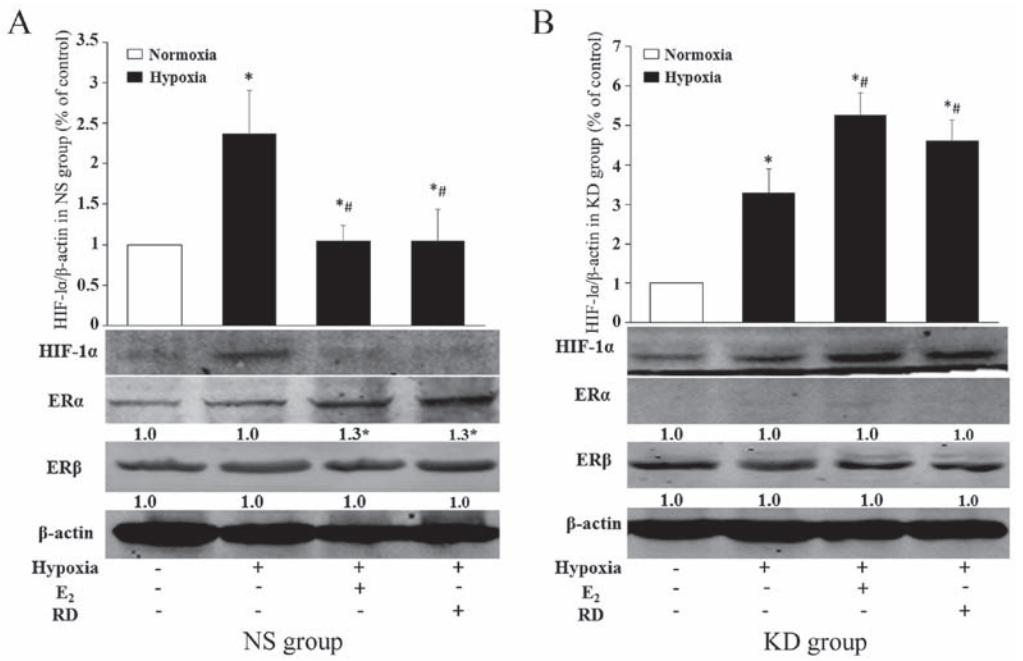


Figure 5. Comparison of hypoxia-inducible factor-1α (HIF-1α), estrogen receptor α (ERα) and ERβ protein expression in genioglossus myoblasts, relative to β-actin. (A) HIF-1α, ERα and ERβ protein expression in myoblasts in the NS group. (B) HIF-1α, ERα and ERβ protein expression in myoblasts in the KD group. NS, transfected with negative scramble siRNA; KD, transfected with ERα-siRNA. The myoblasts were left untreated or treated with 1 μmol/l 17β-estradiol (E_2) or resveratrol dimer (RD) in hypoxia for 24 h. *P<0.05 vs. normoxia; #P<0.05 vs. hypoxia.

significantly lower in hypoxia than in normoxia (P<0.05), while it was increased after E_2 treatment (P<0.05). As expected, the effects of E_2 were blocked by p38 MAPK inhibitor SB203580 (P<0.05). Total p38 MAPK expression was not affected by the different treatments.

To ascertain the role of ERα in E_2-initiated p38 MAPK activation, we knocked down ERα by siRNA and found that E_2 treatment increased phosphorylation of p38 MAPK in the genioglossus myoblasts, which was blocked by ERα knockdown (Fig. 6B).

Following inhibition of p38 MAPK, E_2 upregulates HIF-1α expression. As shown in the Fig. 7, hypoxia induced the HIF-1α protein expression in the myoblasts (P<0.05), and E_2 reverted it partly. The downregulatory effects of E_2 on HIF-1α coincided

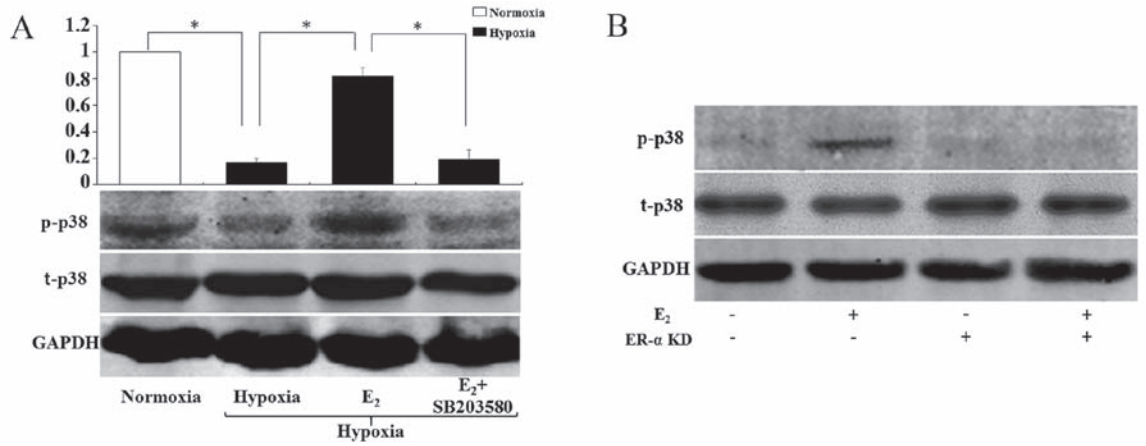


Figure 6. Comparison of p38 MAPK phosphorylation in genioglossus myoblasts, relative to β-actin. (A) Comparison of p38 MAPK phosphorylation. The myoblasts were treated with 1 μmol/l 17β-estradiol (E_2), or 1 μmol/l E_2 and 10 μmol/l SB203580, or vehicle, and then incubated in normoxia or hypoxia for 24 h. (B) Comparison of p38 MAPK phosphorylation in the myoblasts and estrogen receptor α (ERα)-KD ones. The myoblasts and ERα-KD ones were treated with 1 μmol/l E_2 or vehicle in normoxia for 24 h. The experiments were repeated three times with similar results and a representative blot is presented. *P<0.05.

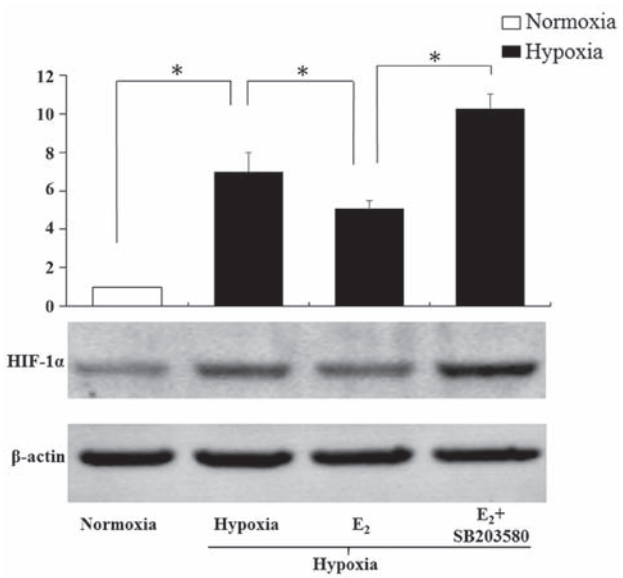


Figure 7. Comparison of hypoxia-inducible factor-1α (HIF-1α) protein expression in genioglossus myoblasts. The myoblasts were treated with vehicle-dimethyl sulfoxide (DMSO), or 1 μmol/l 17β-estradiol (E_2), or 1 μmol/l E_2 and 10 μmol/l SB203580, and then incubated in normoxia or hypoxia for 24 h. Total protein was extracted and HIF-1α and β-actin were analyzed. *P<0.05.

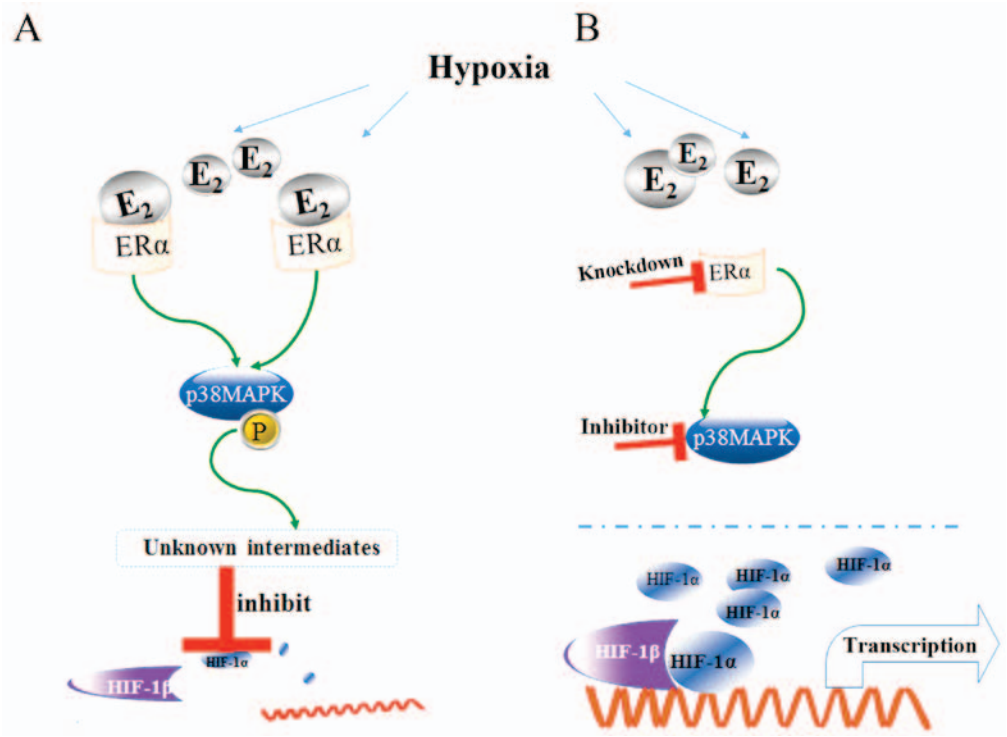
with the former results (Fig. 5A). However, after co-treatment with E_2 and SB203580, HIF-1α protein expression was increased significantly (P<0.05). These results indicated that p38 MAPK pathways were essential in the inhibitory effect of E_2 on HIF-1α in the genioglossus myoblasts.

Discussion

It is documented that the biological effect of estrogen is mediated by ERs. Conventional ERs are transcription factors regulating gene expression, and these mechanisms may be mediated by a series of signaling molecules in several cell types. HIF-1α has been reported to be involved in the hormonal regulation of genes (28,29). In addition, our previous studies demonstrated that downregulation of HIF-1α may be a pivotal explanation for the protective effects of estrogen on the genioglossus in CIH rats (12,13). Yet, hormonal HIF-1α regulation has not been clarified. A clear expression of ERα and weak expression of ERβ in rat genioglossus muscles were detected in our previous study (unpublished data). Furthermore, the expression of ERα, but not ERβ, was found to be regulated by E_2 in the genioglossus of rats (30). In the present study, we revealed that the inhibitory effect of E_2 or RD on HIF-1α expression was ERα-dependent and extended the association of E_2/ERα with HIF-1α. The discovery of E_2- or RD-mediated HIF-1α regulation contributes to our future understanding of the molecular mechanisms underlying OSAHS.

Previous studies have described an association between E_2/ERα and HIF-1 expressions in various organs or cell types. For example, Xu *et al* reported that estrogen treatment reduced the expression levels of HIF-1α and vascular endothelial growth factor (VEGF) and improved the metabolic syndrome in periaortic and intra-abdominal fat in ovariectomized rats (31). Miyauchi *et al* suggested that HIF-1α protein was stabilized in the absence of estrogen but destabilized by treatment with E_2 under hypoxia in osteoclasts, and ERα was required for E_2-dependent HIF-1α destabilization (32). Rzemieniec *et al* confirmed a pivotal involvement of ERα, but not ERβ or the recently identified membrane ER G-protein-coupled receptor 30 (GPER), in the neuroprotective potential of raloxifene, a type of selective estrogen receptor modulator (SERM), against hypoxia-induced damage of mouse hippocampal cells (33). Our results showed that both E_2 and RD inhibited HIF-1α expression in genioglossus myoblasts in a hypoxic condition, and these effects were eliminated by ERα knockdown. Therefore, we showed that ERα plays a crucial role in the downregulatory effect of E_2 and RD on HIF-1α. As for RD, although it exhibited higher binding affinity for ERβ than ERα in our previous study, the downregulatory effect on HIF-1α in hypoxia is also mediated by ERα.

The expression of hypoxia-regulated HIF-1α has been described to be altered along with time (34,35). Therefore, we detected the HIF-1α expression after 24, 48 and 72 h in



Sigure 8. Schematic model illustrating the steps in the 17β-estradiol (E_2)-mediated inhibition of hypoxia-inducible factor-1α (HIF-1α) expression in mouse genioglossus myoblasts. (A) Even in hypoxia, HIF-1α is continuously inhibited by E_2. (B) The inhibitory effects of E_2 were cancelled by estrogen receptor α (ERα) silencing or a p38 MAPK inhibitor.

the present study and observed a similar variation pattern of HIF-1α expression at those three time-points (data not shown). In our study, the protein expression of ERα and ERβ was detected as control. In the NS group, both E_2 and RD markedly activated ERα expression. It can be deduced that the transcriptionally active state of ERα increased through binding to ligands. As shown in Figs. 4 and 5, E_2 or RD inhibited the hypoxia-induced overexpression of HIF-1α, while ERα knockdown prevented this E_2- or RD-dependent HIF-1α suppression. Actually, the expression of HIF-1α was slightly upregulated in the absence of ERα, which may be explained by the roles of other ERs. ERβ is acknowledged to bind to the same nucleotides for DNA contacts, estrogen-responsive element (ERE), with ERα, but induce different structural changes of DNA with the help of ligands and cofactors, and thus display different transcriptional responses (36). In addition, some studies suggest that GPER activates a network of transduction pathways involving the epidermal growth factor receptor (EGFR), the intracellular cyclic AMP (cAMP), the mitogen-activated protein kinase (MAPK) cascade, and calcium mobilization (37-39). De Francesco *et al* reported that E_2 upregulated the expression of HIF-1 at both the mRNA and protein levels via GRER and the EGFR/ERK/c-fos transduction pathway in breast cancer cells and cancer-associated fibroblasts (40). The expression of GPER was not monitored in our studies, but invariable expression of ERβ was observed in both the NS and KD group. As such, it is not unreasonable to suspect that ERβ or GPER could make a contribution to inducing HIF-1α expression in the absence of ERα in myoblasts. Clearly, further study on the relationship of HIF-1α and ERs is required.

Some investigators support that HIF-1α expression following estrogen excess or deficiency is tissue-specific. Studies involving E_2/ERα and HIF-1 expression in skeletal muscles, to our knowledge, are much less than those regarding estrogen reproductive tissues, such as breast, ovary and uterus. This is the first study to show that ERα is responsible for the inhibitory effects of E_2 and RD on HIF-1α in genioglossus myoblasts.

To shed more light on the mechanism underlying the inhibitory effect of E_2 on HIF-1α, we extended the association of E_2/ERα with HIF-1α to include an interaction with p38 MAPK signaling pathways. E_2 is known to be involved in the activation of p38 MAPK signaling pathways (28,41-43). There are many studies that have indicated a role of these two pathways in the regulation of HIF-1α transactivity and synthesis (43,44). We observed that exposure of genioglossus myoblasts to hypoxia for 24 h significantly inhibited the phosphorylation of p38 MAPK, illustrating that inactivity of p38 MAPK signaling pathways may be related to HIF-1α expression. In the present study, treatment of myoblasts with E_2 led to the generation of phosphorylated p38 MAPK, but the phosphorelation effect was blunted in the absence of ERα. To determine whether the signaling pathways lie on the upstream of HIF-1, we observed the effects of p38 MAPK inhibitor SB203580 on the expression of HIF-1α. The induction of p38 MAPK phosphorylation by E_2 was blocked by SB203580. Moreover, p38 MAPK inhibitor induced the expression of HIF-1α even in the existence of E_2, indicating that these signaling molecules were involved in the inhibitory effect of E_2/ERα on HIF-1α expression. To sum up, these results suggest that activation of the p38 MAPK pathways plays an important role in the inhibitory effect of E_2 on HIF-1α expression.

In conclusion, the present study demonstrated that both E_2 and RD inhibited the overexpression of HIF-1α in genioglossus myoblasts under hypoxic condition. ERα knockdown prevented the suppression, indicating that ERα may be responsible for these inhibitory effects. Moreover, activation of the p38 MAPK pathways may play an important role in the inhibitory effect of E_2 on HIF-1α expression (Fig. 8).

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