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

YUANYUAN LI¹, YUEHUA LIU¹,², YUN LU² and BINGJIAO ZHAO²

¹Department of Orthodontics, School and Hospital of Stomatology, Tongji University, Shanghai Engineering Research Center of Tooth Restoration and Regeneration, Shanghai 200072; ²Department of Orthodontics, Shanghai Stomatological Hospital, Shanghai 200001, P.R. China

Received December 19, 2016; Accepted August 23, 2017

DOI: 10.3892/ijmm.2017.3123

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 (E₂) 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 E₂ and RD on the viability of the cells. Cells in different groups were treated with different agents (E₂, or RD, or E₂ 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 E₂ 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 E₂ activated p38 MAPK pathways to inhibit HIF-1α expression. On the whole, ERα may be responsible for downregulation of HIF-1α by E₂ 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 (E₂) exerts protective effects of fatigue resistance on the genioglossus in CIH rats (13,14), and these effects were coincident with the downregulation of...
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 cytotoxity in pre-experiments. Studying the effects of RD on HIF-1α and comparing the results with those obtained from E2 may be the first move to explore the possibility of RD replacing E2 in the medical therapy for OSAHS.

In the present study, we isolated genioglossus myoblasts and silenced ERα to investigate the effect of E2 and RD on HIF-1α and the underlying mechanism. Our study may aid to elucidate the molecular mechanism of E2 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 bicinechonic 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 2x10⁴ cells/ml. After reaching 60% confluence, plated cells were fixed with paraformaldehyde [BSA, dissolved in phosphate-buffered saline (PBS)] for 1 h at room temperature, and incubated with 1:500 DAB; Beyotime Institute of Biotechnology) kit and RD on [HIF-1α and comparing the results with those obtained from E2 may be the first move to explore the possibility of RD replacing E2 in the medical therapy for OSAHS.

In the present study, we isolated genioglossus myoblasts and silenced ERα to investigate the effect of E2 and RD on HIF-1α and the underlying mechanism. Our study may aid to elucidate the molecular mechanism of E2 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 bicinechonic 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 2x10⁴ cells/ml. After reaching 60% confluence, plated cells were fixed with paraformaldehyde [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 diamino-nobenzide (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'-GGAATATTGTTGAAGCACAAGC-3' and reverse, 5'-GCTTGTGCTTCAACATTTCTCC-3' sequences. The scrambled fragment was inserted as the control: ERα scrambled (ERα-NS) forward, 5'-GGTTCCTCGGAACGTGACCG-3' and reverse, 5'-ACGTGACACGTTCGGAGAAC-3'. 293T cells (obtained from the Type Culture
Collection of the Chinese Academy of Sciences, Shanghai, China) were seeded onto 6-well plates at a density of 9×10^4 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 2×10^4 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, 9 µl water, 0.8 µl forward primer, 0.8 µl reverse primer, and 0.4 µl SYBR Premix Ex Taq.
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.

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 E2 and RD on genioglossus myoblast viability were determined by MTT assay (Fig. 3). The exposure of myoblasts to 10 µM E2 resulted in a significant decrease in cell viability under both normoxic and hypoxic conditions, while at other lower concentrations E2 had no inhibitory effect within 72 h. The same trend was found in
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
significantly lower in hypoxia than in normoxia (P<0.05), while it was increased after E₂ treatment (P<0.05). As expected, the effects of E₂ 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 E₂-initiated p38 MAPK activation, we knocked down ERα by siRNA and found that E₂ treatment increased phosphorylation of p38 MAPK in the genioglossus myoblasts, which was blocked by ERα knockdown (Fig. 6B).

Following inhibition of p38 MAPK, E₂ upregulates HIF-1α expression. As shown in the Fig. 7, hypoxia induced the HIF-1α protein expression in the myoblasts (P<0.05), and E₂ reverted it partly. The downregulatory effects of E₂ on HIF-1α coincided
with the former results (Fig. 5A). However, after co-treatment with E2 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 E2 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 E2 in the genioglossus of rats (30). In the present study, we revealed that the inhibitory effect of E2 or RD on HIF-1α expression was ERα-dependent and extended the association of E2/ERα with HIF-1α. The discovery of E2- or RD-mediated HIF-1α regulation contributes to our future understanding of the molecular mechanisms underlying OSAHS.

Previous studies have described an association between E2/ERα and HIF-1 expression 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 E2 under hypoxia in osteoclasts, and ERα was required for E2-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 E2 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 E2 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

![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 (E2), or 1 µmol/l E2 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 E2 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.](image)

![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 (E2), or 1 µmol/l E2 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.](image)
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 E2 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, E2 or RD inhibited the hypoxia-induced overexpression of HIF-1α, while ERα knockdown prevented this E2- or RD-dependent HIF-1α suppression. Actually, the expression of HIF-1α was slightly upregulated in the absence of ERs, 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 E2 upregulated the expression of HIF-1α 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 E2/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 E2 and RD on HIF-1α in genioglossus myoblasts.

To shed more light on the mechanism underlying the inhibitory effect of E2 on HIF-1α, we extended the association of E2/ERα with HIF-1α to include an interaction with p38 MAPK signaling pathways. E2 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 E2 led to the generation of phosphorylated p38 MAPK, but the phosphorylation 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 E2 was blocked by SB203580. Moreover, p38 MAPK inhibitor induced the expression of HIF-1α even in the existence of E2, indicating that these signaling molecules were involved in the inhibitory effect of E2/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 E2 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).

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China, 81271192 (2012). We are grateful to Professor X. Sun and Dr C. Zhong (School of Pharmacy, Fudan University, Shanghai, China) for supply of resveratrol dimers.

References

1. Eckert DJ and Malhotra A: Pathophysiology of adult obstructive sleep apnea. Proc Am Thorac Soc 5: 144-153, 2008.
2. Douglass NY and Polo O: Pathogenesis of obstructive sleep apnea/hypopnea syndrome. Lancet 344: 653-655, 1994.
3. Owens RL, Eckert DJ, Yeh SY and Malhotra A: Upper airway function in the pathogenesis of obstructive sleep apnea: A review of the current literature. Curr Opin Pulm Med 14: 519-524, 2008.
4. Sérieis FJ, Simonneau SA, St Pierre S and Marc I: Characteristics of the genioglossus and muscle uvulae in sleep apnea hypopnea syndrome and in snorers. Am J Respir Crit Care Med 153: 1870-1874, 1996.
5. Bradford A, McGuire M and O’Halloran KD: Does episodic hypoxia affect upper airway dilator muscle function? Implications for the pathophysiology of obstructive sleep apnea. Respir Physiol Neurobiol 147: 232-254, 2005.
6. Malhotra A, Pillar G, Fogel RB, Beauregard J, Edwards JK, Slamowitz DI, Shea SA and White DP: Genioglossus but not palatal muscle activity relates closely to pharyngeal pressure. Am J Respir Crit Care Med 162: 1058-1062, 2000.
7. Cai Z, Manalo DJ, Wei G, Rodriguez ER, Fox-Talbot K, Lu H, Bradford A, McGuire M and O’Halloran KD: Does episodic hypoxia affect upper airway dilator muscle function? Implications for the pathophysiology of obstructive sleep apnea. Respir Physiol Neurobiol 147: 232-254, 2005.
8. Yuan G, Nanduri J, Bhasker CR, Semenza GL and Prabhakar NR: p38 MAPK pathways may play an important role in the inhibitory effect of $E_2$ on HIF-1α expression (Fig. 8).
9. Liu Y, Liu Y and Li Y: Comparison of natural estrogens and synthetic derivative on genioglossus function and estrogen receptors expression in rats with chronic intermittent hypoxia. J Steroid Biochem Mol Biol 140: 71-79, 2014.
10. Solakidi S, Psarra AM and Sekeris CE: Differential distribution of glucocorticoid and estrogen receptor isoforms: Localization of GRβ and ERα in nuclei of and GRα and ERβ in the mitochondria of human osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cell lines. J Musculoskeletal Neuronal Interact 7: 240-245, 2007.
11. Aschim EL, Saether T, Wiger R, Grotmol T and Haugen TB: Differential distribution of splice variants of estrogen receptor beta in human testicular cells suggests specific functions in spermatogenesis. J Steroid Biochem Mol Biol 92: 97-106, 2004.
12. Cousse JF, Lindsey J, Grandien K, Gustafsson JA and Korach KS: Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Biol Reprod 138: 4613-4621, 1997.
13. Kalbe C, Mau M, Wollenhaupt K and Rehfeldt C: Evidence for estrogen receptor alpha and beta and expression in skeletal muscle of pigs. Histochem Cell Biol 127: 95-107, 2007.
14. Veasey SC, Guilleminault C, Strohl KP, Sanders MH, Ballard RD and Magalang UJ: Medical therapy for obstructive sleep apnea: A review by the Medical Therapy for Obstructive Sleep Apnea Task Force of the Standards of Practice Committee of the American Academy of Sleep Medicine. Sleep 29: 1036-1044, 2006.
15. Bixler EO, Vgontzas AN, Lin HM, Ten Have T, Rein J, Vela-Bueno A and Kales A: Prevalence of sleep-disordered breathing in women: Effects of gender. Am J Respir Crit Care Med 163: 608-613, 2001.
16. Pickett CK, Regensteiner JG, Woodard WD, Hagerman DD, Weil JV and Moore LG: Progestin and estrogen reduce sleep-disordered breathing in postmenopausal women. J Appl Physiol (1985) 66: 1656-1661, 1989.
17. Popovic RM and White DP: Upper airway muscle activity in normal women: Influence of hormonal status. J Appl Physiol (1985) 84: 1055-1062, 1998.
18. Brown GB, Yang L and Baudry M: The pros and cons of phytoestrogens. Front Neuroendocrinol 31: 400-419, 2010.
19. Zhong C, Zhu J, Chang J and Sun X: Concise total syntheses of (+)-isopaucafloral F, (+)-quadruaruginil A, and (+)-pillifolin. Tetrahedron Lett 258: 2815-2817, 2011.
20. Bi R, Brouttman G, Foy MR, Thompson RF and Baudry M: The transforming kinase of mice and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. Proc Natl Acad Sci USA 97: 3602-3607, 2000.
21. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R and Santen RJ: Linkage of rapid estrogen action to MAPK activation by ERα-β: Signal transduction and She pathway activation. Mol Endocrinol 16: 116-127, 2002.
22. Chen CC, Lee WR and Safe S: Egr-1 is activated by 17beta-estradiol in MCF-7 cells by mitogen-activated protein kinase-dependent phosphorylation of ELK-1. J Cell Physiol 193: 1063-1074, 2004.
23. Kazi AA and Koos RD: Estrogen-induced activation of hypoxia-inducible factor-lalpha, vascular endothelial growth factor factor expression, and edema in the uterus are mediated by the phosphatidylinositol 3-kinase/Akt pathway. Endocrinology 148: 2437-2447, 2007.
24. Yun SP, Lee MY, Ryu JM, Song CH and Han HJ: Role of HIF-α/alphag- VEGF in human mesenchymal stem cell proliferation by 17beta-estradiol: Involvement of PKC, PI3K/Akt, and MAPKs. Am J Physiol Regul Integr Comp Physiol 273: R1335-R1341, 2006.
25. Hou XY, Jia SS and Liu YH: 17beta-Estradiol accentuates contractility of rat genioglossus muscle via regulation of estrogen receptor alpha. Arch Oral Biol 55: 309-317, 2010.
26. Xu J, Xiang Q, Lin G, Fu X, Zhou K, Jiang P, Zheng S and Wang T: Estrogen improved metabolic syndrome through down-regulation of VEGF and HIF-1α to inhibit hypoxia of periaortia and intra-abdominal fat in ovariectomized female rats. Mol Biol Rep 39: 8177-8185, 2012.
27. Miyauchi Y, Sato Y, Kobayashi T, Yoshida S, Mori T, Kanagawa H, Katsuyama E, Fujie A, Hao W, Miyamoto K, et al.: HIF-1α is required for osteoclast activation by estrogen deficiency in postmenopausal osteoporosis. Proc Natl Acad Sci USA 110: 16568-16573, 2013.
28. Rzemieniec J, Litwa E, Wnuk A, Lason W, Gofas A, Krzepkowski W and Kajta M: Neuroprotective action of raloxifene against hypoxia-induced damage in mouse hippocampal cells depends on ERβ but not ERα or GPR30 signalling. J Steroid Biochem Mol Biol 146: 26-37, 2015.
29. Cats C, van Heeringen P, de Zeeuw D, Hattink C, Verheijden M, et al.: Raloxifene against hypoxia-induced damage in mouse hippocampal cells depends on ERβ but not ERα or GPR30 signalling. J Steroid Biochem Mol Biol 146: 26-37, 2015.
30. Camps C, Saini HK, Mole DR, Choudhry H, Rezco M, Guerrera-Assunção JA, Tian YM, Bufla FM, Harris AL, Hatziegorgiou AG, et al.: Integrated analysis of miRNA and mRNA expression profiles and association with HIF binding reveals the complexity of microRNA expression regulation under hypoxia. Mol Cell 13: 28, 2014.
31. Chung HY, Lee SJ, Lee JM, Huh S, Kim HK, Kwon OH, Lim HJ, Oh EJ, Kim TJ, O TM, et al.: Expression patterns of HIF-lalpha under hypoxia in vascular smooth muscle cells of venous malformations. Ann Plast Surg 75: 332-3, 2015.
36. Yi P, Driscoll MD, Huang J, Bhagat S, Hilf R, Bambara RA and Muyan M: The effects of estrogen-responsive element- and ligand-induced structural changes on the recruitment of cofactors and transcriptional responses by ER alpha and ER beta. Mol Endocrinol 16: 674-693, 2002.

37. Filardo EJ, Quinn JA, Bland KI and Frackelton AR Jr: Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14: 1649-1660, 2000.

38. Thomas P, Pang Y, Filardo EJ and Dong J: Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146: 624-632, 2005.

39. Revankar CM, Cimino DF, Sklar LA, Arterburn JB and Prossnitz ER: A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307: 1625-1630, 2005.

40. De Francesco EM, Pellegrino M, Santolla MF, Lappano R, Ricchio E, Abonante S and Maggiolini M: GPER mediates activation of HIF1α/VEGF signaling by estrogens. Cancer Res 74: 4053-4064, 2014.

41. Guzeloglu Kayisli O, Kayisli UA, Luleci G and Arici A: In vivo and in vitro regulation of Akt activation in human endometrial cells is estrogen dependent. Biol Reprod 71: 714-721, 2004.

42. Linford NJ, Yang Y, Cook DG and Dorsa DM: Neuronal apoptosis resulting from high doses of the isoflavone genistein: Role for calcium and p42/44 mitogen-activated protein kinase. J Pharmacol Exp Ther 299: 67-75, 2001.

43. Salceda S, Beck I, Srinivas V and Caro J: Complex role of protein phosphorylation in gene activation by hypoxia. Kidney Int 51: 556-559, 1997.

44. Mazure NM, Chen EY, Laderoute KR and Giaccia AJ: Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood 90: 3322-3331, 1997.