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Keratinocyte growth factor protects endometrial cells from oxygen glucose deprivation/re-oxygenation via activating Nrf2 signaling

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ABSTRACT

Oxygen and glucose deprivation (OGD)-re-oxygenation (OGDR) exposure to endometrial cells mimics ischemia-reperfusion injury. The present study tests the potential effect of keratinocyte growth factor (KGF) on the process. We show that KGF receptor KGFR is expressed in human endometrial T-HESC cells and primary murine endometrial cells. KGF pre-treatment protected endometrial cells from OGDR, inhibiting cell viability reduction and cell death. KGF attenuated OGDR-induced programmed necrosis in endometrial cells. Significantly, KGF activated Nrf2 signaling, causing Nrf2 Ser-40 phosphorylation, protein stabilization, nuclear translocation to promote anti-oxidant gene (*HO1*, *NOQ1* and *GCLC*) expression. Nrf2 silencing (by targeted shRNAs) or CRISPR/Cas9 knockout almost abolished KGF-induced endometrial cell protection against OGDR. Furthermore, KGF activated Akt-mTOR signaling in endometrial cells. Whereas Akt-mTOR inhibitors (LY294002, AZD2014 and RAD001) abolished KGF-induced Nrf2 activation and anti-OGDR cytoprotection. Together, KGF protects endometrial cells from OGDR via activating Akt-mTOR-Nrf2 signaling.

1. Introduction

Postpartum hemorrhage is a common complication in obstetrics clinic [1,2,3], which causes ischemic damage to endometrium [1,2,3]. Ischemia is often accompanied with re-perfusion, causing further damage to endometrium [1,2,3]. Ischemia-reperfusion induces reactive oxygen species (ROS) production [4,5,6], and lipid peroxidation, oxidative stress, DNA damage and eventually endometrial cell death [1,2,3,4,5,6]. Oxygen and glucose deprivation (OGD)-re-oxygenation (OGDR) exposure to endometrial cells [7] mimics the pathological condition of ischemia-reperfusion injury [8,9,10,11].

Keratinocyte growth factor (KGF) binds to its receptor KGF receptor (KGFR) [12,13]. Thereafter, KGFR will be phosphorylated, recruiting FGF receptor substrate 2 (FRS-2) and other adaptor proteins [14] to activate downstream signaling cascades [14,15,16,17]. PI3K-Akt-mTOR and Erk signaling activation [14] can promote cell survival, proliferation and migration [14]. The potential effect of KGF, and its receptor KGFR, in endometrial cells has not been studied thus far.

The transcription factor Nrf2 (nuclear-factor-E2-related factor 2) regulates expression of multiple key anti-oxidant genes [18,19,20]. In the resting condition, Nrf2 stays in the cytoplasm through binding to its suppressor Keap1 [18,19,20]. The latter will promotes Nrf2 ubiquitination and proteasomal degradation [18,19,20]. Once activated, Nrf2 departs from Keap1, leading to its stabilization, accumulation and translocation to nuclei [18,19,20], where Nrf2 binds to antioxidant responsive element (ARE) [18,19,20]. Nrf2-ARE binding leads to transcription and expression of key anti-oxidant genes, including *heme oxygenase-1* (*HO1*), *NAD(P)H quinone oxidoreductase 1* (*NQO1*) and *γ-glutamyl cysteine ligase catalytic subunit* (*GCLC*) [18]. The present study shows that KGF, via activating Nrf2 signaling, protects endometrial cells from OGDR.

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2. Materials and methods

2.1. Chemical and reagents

KGF and puromycin were purchased from Sigma-Aldrich Chemicals (St Louis, Mo). LY294002, AZD2014 and Everolimus (RAD001) were obtained from Selleck Chemicals (Houston, TX). All reagents for cell culture were purchased from Gibco BRL (Grand Island, NY). The antibody for phosphorylated ("p-") Nrf2 at Ser-40 was provided by Dr. Jiang [21,22]. Other antibodies of the present study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Tech (Suzhou, China).

2.2. Cell culture

The culture of immortalized human endometrial cell line, T-HESC [23], was described in our previous study [7]. Isolation and culture of primary murine endometrial (stromal) cells were described previously as well [7]. Briefly, the fresh mouse uterine tissues were incubated with trypsin-EDTA and collagenase I (Sigma-Aldrich) solution. Next, the uterine tissues were transferred to DMEM/Hams F-12 nutrient plus 10% FBS. The epithelial cells were abandoned using gravity sedimentation. The primary endometrial stromal cells were pelleted, and re-suspending in the complete medium. The protocol of using primary cells was approved by the Ethics Board of Changzhou Second People's Hospital.

2.3. Cell viability assay

Cells were initially seeded onto the96-well tissue culture plates (3×10^3 cells per well). The cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) was applied to test cell viability, according to the standard procedure [7]. The CCK-8 optic density (OD) at 450 nm was recorded.

2.4. Trypan blue staining of "dead" cells

Cells were initially seeded onto the24-well tissue culture plates (2×10^4 cells per well). As described previously [7], following the treatment, only the dead cells were positive for trypan blue (Sigma), and its percentage (%) was recorded using an automatic cell counter (Roche, Shanghai, China).

2.5. Lactate dehydrogenase (LDH) assay

Cells were initially seeded onto the12-well tissue culture plates (5×10^4 cells per well). The release of LDH to the cell medium is a well-known marker of cell necrosis [24], which was quantified by a two-step LDH detection kit (Promega, Shanghai, China) [7].

2.6. OGD/re-oxygenation (OGDR)

OGDR procedure was described previously [7]. Briefly, endometrial cells were first placed into an airtight chamber (95% N_2/5% CO_2) for 4 h (mimicking oxygen glucose deprivation). Afterwards, the endometrial cells were returned back to the complete medium and re-oxygenated.

2.7. Western blotting assay

The total cell lysates were achieved via incubating the endometrial cells in the RIPA lysis buffer (Beyotime Biotechnology, Wuxi, China). The lysates proteins (40 μg per treatment of each lane) were separated by 10–12% SDS-PAGE gels, and transferring to the polyvinylidene difluoride (PVDF) blot [25]. The detailed protocol of Western blotting assay and data quantification were described previously [22,26]. The protein lysates of primary retinal ganglion cells (RGCs) were provided by Dr. Jiang, as negative control with no KGFR expression [15]. Assaying of nuclear fraction proteins was described previously [22].

2.8. Mitochondrial immunoprecipitation (Mito-IP)

After the indicated KGF treatment, T-HESC cells were harvested by the described lysis buffer [7]. After centrifugation, the supernatants were collected as the cytosolic fractions. The pellets were then re-suspended to achieve mitochondrial fraction lysates. The quantified mitochondrial lysates (300 μg per sample) were pre-cleared, and incubated with anti-cyclophilin-D (Cyp-D) antibody (Santa Cruz Biotech). The mitochondrial complex was then captured by the protein G-Sepharose beads (Sigma). Cyp-D-p53-ANT-1 association was tested by Western blotting assay.

2.9. Mitochondrial depolarization assay

Following mitochondrial depolarization ("ΔΨ"), the mito-dye JC-1 (Sigma) will aggregate to form the green monomers [27]. Cells were initially seeded onto the24-well tissue culture plates (2×10^4 cells per well). Assay of mitochondrial depolarization by the JC-1 protocol was discussed previously [7], JC-1 fluorescence OD was examined at 530 nm.

2.10. ROS detection

As previously described [7], the fluorescent dye DCFH-DA (2′,7′-dichlorofluorescein diacetate) was applied to examine cellular ROS intensity. Cells were initially seeded onto the24-well tissue culture plates (2×10^4 cells per well). After the applied treatment, cells were incubated with DCFH-DA (100 μM, Invitrogen) for 60 min. The DCF fluorescence intensity at 530 nm was recorded.

2.11. Lipid peroxidation assay

As described in our previous study [7], cellular lipid peroxidation was evaluated by the TBAR (thiobarbituric acid reactive substances) assay [28]. Briefly, for each treatment, 20 μg total cell lysates were mixed with 20% of acetic acid and thiobarbituric acid solution. After heating, the mixture was centrifuged, and the red pigment dye in the supernatant was examined by the microplate reader [28].

2.12. qRT-PCR

The total cellular RNA was extracted [7]. The ABI Prism 7600 Fast Real-Time PCR system was utilized to perform the quantitative real time-PCR (qRT-PCR) assay. Melt curve analysis was always performed to calculate product melting temperature. mRNA primers for both murine and human Nrf2 pathway genes were provided by Dr. Jiang [15,22,29]. mRNA primers for KGFR were provided by Dr. Cao [14]. *Glyceraldehyde-3-phosphatedehydrogenase* (*GAPDH*) *mRNA* was tested as the reference gene, and the $2^{-\Delta\Delta Ct}$ method was utilized for the quantification of targeted mRNA.

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