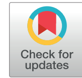
Theriogenology 115 (2018) 9–15

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Hypoxia-dependent accumulation of hypoxia-inducible factor-1 alpha induces transient cell cycle arrest in porcine trophectoderm cells



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ARTICLE INFO

Article history:
Available online 14 April 2018

Keywords:
Hypoxia
Trophectoderm
HIF-1
Cell cycle
G_1/S transition

ABSTRACT

In the uterine environment, the pre-implantation embryo adapts to low oxygen concentrations through intracellular responses including modification of gene expression, progression via the cell cycle and metabolism. In this study, we determined mechanisms underlying the adaptation of pig embryos to oxygen deficiency in the maternal-conceptus microenvironment in *in vitro* experiments using our established porcine trophectoderm (pTr) cells in culture. The transition from G_1 to S phase in pTr cells was reduced in response to 2% oxygen during a short period (<24 h), and the hypoxia-induced G_1 arrest was reversible during prolonged hypoxia exposure. Acute hypoxia up-regulated expression of transcription factors p21 and p27 and down-regulated cell cycle regulators associated with the G_1/S phase transition including cyclin D1, cyclin E1 and E2F1 *mRNAs* and proteins. Furthermore, hypoxia exposure for 24 h markedly increased the abundance of HIF-1α protein. Even under acute hypoxia, by HIF-1α silencing reduced the hypoxia-induced transient G_1 arrest and expression of p21 and p27 genes was restored. Contrary to acute hypoxia, the accumulation of HIF-1α protein decreased as the length of the hypoxic period increased. Overall results of the present study suggest that increases in HIF-1α are responsible for initial response to hypoxia that results in a transient cell cycle arrest in pTr cells and cell cycle progression is restored by increasing degradation of HIF-1α during prolonged hypoxia. These findings advance understating of cellular adaptation of developing pre-implantation porcine conceptuses to hypoxic stress.

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1. Introduction

Hypoxia, a reduction in the normal level of tissue oxygen tension, is found in many physiological (e.g., exercise, embryonic development, underwater diving, or high altitude) and pathological (e.g., inflammation, solid tumor formation, lung disease, or myocardial infarction) conditions. Cellular hypoxia occurs in tissue that is > 100–200 μm away from a functional blood supply. Low oxygen tension in the uterine lumen is a physiologically relevant environmental stress because the pre-implantation embryo completely depends upon diffused oxygen from maternal uterine tissue. Furthermore, during the time of implantation, oxygen tension in the uterine environment is 5–8% or less; therefore, hypoxic and even anoxic conditions confront the conceptus (embryo and its extra-embryonic membranes) [1,2]. If pre-implantation embryos are unresponsive to such stressful oxygen tension, they will fail to survival and develop and, ultimately will die. Consequently, for early embryos, as they progress from the maternal oviduct to the uterus, the ability to respond and adapt to hypoxic stress is necessary for their survival and for development of conceptus. This may be especially critical in livestock species such as the pig which has a non-invasive implantation resulting in a protracted pre-attachment phase compared to other species [3].

The classical cellular response and adaptation to oxygen deficiency includes changes in metabolic pathways [4]. The adaptation of cells to hypoxic stress is mediated principally via the transcription factor hypoxia-inducible factor-1 (HIF-1) [5]. Active HIF-1 is a heterodimeric complex consisting of two subunits: a constitutively expressed β subunit (also termed aryl hydrocarbon nuclear translocator; ARNT) and an inducible α subunit. Generally, the *HIF-1α* gene is expressed ubiquitously even in normoxic tissues, but HIF-1α proteins accumulate in cells under hypoxic conditions. In normoxic

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https://doi.org/10.1016/j.theriogenology.2018.04.016
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tissues, *de-novo* synthesized HIF-1α undergoes post-translational modifications (including hydroxylation and ubiquitination) and then the modified protein is rapidly degraded in the proteasome [6–8]. On the other hand, low oxygen concentrations increase the stability of HIF-1α protein [6–8].

Hypoxia can alter cell cycle progression through HIFs or other hypoxia-induced transcription factors and their putative transcriptional targets involved in cell cycle regulation. For example, hypoxia induces a G_1/S arrest in cells and the effect of hypoxia on cell cycle regulation is dependent on HIF-1α-mediated expression of the cell-cycle regulators such as p21 [9] and p27 [10]. However, there are no reports of cell cycle regulation in response to hypoxic stress in porcine trophectoderm cells and very limited information on the roles of hypoxic-induction of HIF-1α in developing porcine embryos. Therefore, the aim of this study was 1) to determine whether reduced oxygen tension affects cell cycle progression and expression of cell cycle regulator genes in porcine trophectoderm (pTr) cells; 2) to investigate whether the hypoxia-induced pTr cell cycle regulation is related to HIF-1α accumulation; and 3) to investigate regulation of HIF-1α accumulation and cell cycle progression in pTr cells under acute and chronic hypoxic stress.

2. Materials and methods

2.1. Cell culture

Porcine trophectoderm (pTr) cell line established previously by Ka et al. [11] using conceptuses collected from Day 12 pregnant gilts was used. The pTr cells were cultured and used in the present *in vitro* studies as described previously [11]. Monolayer cultures of pTr cells were grown in DME/F12 1:1 medium containing 10% FBS. For normoxic oxygen conditions (21% O_2) cells were incubated in a standard humidified incubator at 37 °C and 5% CO_2. The hypoxic conditions (10%; moderate hypoxia, or 2%; severe hypoxia) involved culturing pTr cells in a humidified incubator under an atmosphere of 10% O_2, 85% N_2 and 5% CO_2 (for moderate hypoxia) or 2% O_2, 93% N_2 and 5% CO_2 (for severe hypoxia) at 37 °C.

2.2. Cell cycle analysis using propidium iodide (PI) staining

To determine the effect of oxygen deficiency on cell cycle progression, pTr cells were exposed to different oxygen tensions (21%, 10% or 2% O_2) prior to cell cycle analyses by flow cytometry. Cells were seeded in 6-well plate and cultured under normoxic or hypoxic culture conditions for various periods of time. The cells were harvested and washed with cold PBS and then fixed with 70% ethanol. Cell pellets were then resuspended in 0.5 ml PBS containing 50 μg/ml propidium iodide (PI) and 100 μg/ml DNase-free RNase. Cell cycle analyses were performed using a flow cytometer (BD Biosciences, San Jose, CA).

2.3. BrdU incorporation analysis

Proliferation assays were conducted using the Cell Proliferation ELISA, BrdU kit (Roche, Basel, Switzerland) according to the manufacturer's recommendations. Briefly, pTr cells were seeded in a 96-well microplate (tissue culture grade, flat bottom) and exposed to normoxic or hypoxic conditions for 24 h. After labeling the pTr cells with BrdU for additional 4 h, they were incubated with *anti*-BrdU-POD working solution for 90 min. The absorbance values of the reaction product were quantified by measuring the absorbance at the 370 nm using an ELISA reader (Bio-Rad, Hercules, CA).

2.4. Transfection of target-specific siRNAs for HIF-1α or PHDs

Potential small interfering RNA target sites for mRNA sequences of porcine HIF-1α or PHD1-3 isoforms were determined using the Invitrogen design program. The pTr cells were treated with a specific HIF-1α or PHDs (three isoforms)-specific siRNA for 16 h or controls that included naïve treatment (no siRNA or Lipofectamine 2000), mock treatment (Lipofectamine 2000 only) or control siRNA, and then the siRNA-transfected or control cells were subjected to hypoxia.

2.5. RNA isolation and cDNA synthesis

Total cellular RNA was isolated from pTr cells exposed to normoxic or hypoxic conditions using Trizol reagent (Invitrogen, Carlsbad, CA) and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Complementary DNA was synthesized using AccuPower® RT PreMix (Bioneer, Daejeon, Korea).

2.6. Quantitative PCR analysis

To confirm that oxygen deficiency has an effect on pTr cell cycle progression, we analyzed mRNA expression of cell cycle regulators associated with the G_1/S transition. Gene expression was determined using the SYBR® Green (Sigma, St. Louis, MO) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR conditions were 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s, 64 °C for 40 s, and 72 °C for 1 min using a melting curve program (increasing the temperature from 55 °C to 95 °C at 0.5 °C per 10 s) and continuous fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the C_T value represented the cycle number, and relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method. We have confirmed that Ct values for porcine *beta-actin* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) genes have remained with low variance under both control and experimental conditions. The *β-actin* and *GAPDH* genes were simultaneously analyzed as the endogenous control to standardize the amount of RNA in each reaction.

2.7. Western blot analyses

Proteins separated by SDS-PAGE were transferred to nitrocellulose. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico, Rockford, IL) and quantified by measuring the light intensity from correctly sized bands using a ChemiDoc EQ system (Bio-Rad, Hercules, CA). A house keeping protein (tubulin) was detected using mouse anti-human monoclonal alpha Tubulin (TU-02) antibody (Santa Cruz Biotechnology CA, USA; catalog number: SC-8035) as a loading control for proteins. Antibodies against p21 (catalog number: 2947), p27 (catalog number: 2552), Cyclin D1 (catalog number: 2922), Cyclin E1 (catalog number: 20808) and E2F1 (catalog number: 3742) were purchased from Cell Signaling Technologies (Beverly, MA). HIF-1α polyclonal antibody (catalog number: PA1-16627) was purchased from Thermo Fisher Scientific (Waltham, MA).

2.8. Statistical analyses

Data from at least three independent experiments were analyzed and presented as least-square means (LSMs) with SEMs. All quantitative data were subjected to least squares analysis of variance (ANOVA) using the General Linear Model procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Data

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