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Expression of hypoxia-inducible factor-1 by trophectoderm cells in response to hypoxia and epidermal growth factor

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ABSTRACT

The low oxygen environment in the uterine environment requires pre-implantation embryos to adapt to oxygen deficiency. Hypoxia-inducible factor (HIF)-1 is a master regulator whereby cells adapt to changes in oxygen concentrations. In addition to hypoxic conditions, non-hypoxic stimuli such as growth factors also activate expression of HIF-1. In this study, the mechanisms underlying low oxygen-dependent and epidermal growth factor (EGF)-dependent expression of HIF-1α were explored using porcine trophectoderm (pTr) cells. The results indicated that expression of *HIF-1α* and *HIF-1β* mRNAs was not affected by low concentrations of oxygen; however, hypoxic conditions markedly increased the abundance of HIF-1α protein, especially in nuclei of pTr cells. Even under normoxic conditions, the abundance of HIF-1α protein increased in response to EGF. This EGF-mediated increase in HIF-1α protein was blocked through inhibition of translation by cycloheximide. The inhibitors LY294002 (PI3K-AKT inhibitor), U0126 (inhibitor of ERK1/2) and rapamycin (mTOR inhibitor) also blocked the ability of EGF to increase HIF-1α protein and to phosphorylate AKT, ERK1/2 and mTOR proteins. Both hypoxia and EGF induced proliferation of pTr cells. This ability of EGF to stimulate proliferation of pTr cells was suppressed by EGFR siRNA, but not HIF-1α siRNA, but a significant decrease in EGF-induced HIF-1α protein occurred when pTr cells were transfected with HIF-1α siRNA. The results of the present study suggest that pTr cells adapt to oxygen deficiency and proliferate in response to an oxygen-dependent HIF-1 system, and that EGF at maternal–conceptus interface can increase the abundance of HIF-1α protein via translational regulation through AKT, ERK1/2 and mTOR signaling cascades.

1. Introduction

The concentrations of oxygen in the oviduct and uterus are 5–8%, or less which suggests a uterine environment during the peri-implantation period that is close to anoxia [1]. Therefore, developing embryos must respond to decreasing oxygen gradients as they progress from the maternal oviduct to the uterine environment. If embryonic cells are unresponsive to this stressful oxygen tension, they fail to survive and develop and, ultimately die. Adaptation of embryos to oxygen deficiency may be especially critical in livestock species such as the pig which has a true non-invasive epitheliochorial type of implantation and a protracted preimplantation phase compare to other species [2].

Within a physiological range, the metabolic adaptation to changes in tissue oxygenation is principally mediated through activation of a ubiquitously expressed transcription factor known as hypoxia-inducible factor-1 (HIF-1) [3]. Active HIF-1 is a heterodimeric complex consisting of two subunits: a constitutively expressed β subunit and an inducible α subunit. Generally, synthesized HIF-1α protein is post-translationally modified and then rapidly degraded within the proteasome under normoxic conditions, while low oxygen concentrations increases HIF-1α protein stability by regulating the degree to which it is hydroxylated [4–6]. Adaptation to altered oxygen concentrations results from the stabilization and activation of HIF-1 which regulates the transcription of a variety of target genes implicated in angiogenesis, metabolic adaptation to hypoxia and cell viability, invasion into the

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extracellular matrix, and vascular remodeling [7,8]. However, there have been no reports of the regulation of the HIF-1 system in porcine trophectoderm in response to low oxygen concentrations, or effects of non-hypoxic factors mediating expression of the HIF-1 system.

Even if altered oxygen tension is the primary mechanism for inducing expression of HIFs, HIFs are capable of dynamic response to a variety of conditions. There is an increasing body of evidence that non-hypoxic stimuli including growth factors [9,10], cytokines [11], vascular hormones [10,12], and viral proteins [13,14] can increase expression of HIF-1 and HIF-1 activity even under normoxic conditions. This suggests that the HIF system in pre-implantation embryos could be an important regulator of embryonic survival or development since the hypoxic maternal uterine environment provides numerous factors including hormones, growth factors and cytokines. Unfortunately, most of the non-hypoxic factors have only recently been identified and little information is available regarding the role(s) of those factors on the HIF-1 system. We determined that EGF acts on pTr cells to enhance survival and development and regulate gene expression [15]. On the basis of those observations, we hypothesized that EGF released from developing conceptus and/or maternal uterine endometrium during early pregnancy may regulate expression of HIFs by pTr cells and that HIF may be a candidate mediator of EGF actions.

Interestingly, unlike hypoxia, the non-hypoxic stimuli-induced mechanisms activating HIF-1 appear to be different depending on stimulus and cell type. For example, in the peri-implantation mouse uterus, Hif1a is up-regulated in the uterine LE in response to progesterone, while Hif2a expression increased in uterine stromal cells in response to estradiol [16]. Determination of the actions of various factors in response to combinations of uterine factors (non-hypoxic stimuli) and hypoxic conditions is paramount to understanding responses of cells of the conceptus to the *in vivo* uterine environment. In the current study, we first investigated hypoxia-dependent activation of the HIF-1 system and focused on mechanisms whereby HIF-1 is regulated by EGF in pTr cells. The purpose of this study was to determine: 1) response of the HIF-1 system in pTr cells to oxygen deficiency; 2) effects of EGF on HIF-1 induction under hypoxic- and non-hypoxic conditions; 3) the signaling cascades involved in EGF-dependent changes in abundance of HIF-1 protein; and 4) the role of EGF-dependent HIF-1 in mediating the actions of EGF in pTr cells.

2. Materials and methods

2.1. Cell culture

The porcine trophectoderm (pTr) cell line established from Day 12 pig conceptuses were cultured in DME/F12 1:1 medium containing 10% FBS. For normoxic oxygen conditions (20–21% O_2) cells were incubated in a standard humidified incubator at 37 °C and 5% CO_2. Under hypoxic conditions cells were cultured in a humidified environment with 5% O_2, 90% N_2 and 5% CO_2 at 37 °C. To test the effects of EGF on HIF-1 activation, cells were treated with recombinant human pro-EGF (R&D Systems, Inc., Minneapolis, MN).

2.2. RNA isolation

Total RNA was isolated from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations.

2.3. Quantitative PCR analysis

The levels of expression of *HIF-1* mRNAs in pTr cells were assessed using quantitative RT-PCR as described previously [15].

2.4. Western blot analyses

Proteins separated by SDS-PAGE and 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).

2.5. Immunofluorescence

Immunofluorescence staining was performed using a rabbit anti-human polyclonal antibody against HIF-1α or HIF-1β (Pierce Biotechnology, Rockford, IL) as described previously [15].

2.6. Proliferation assay

Proliferation assays were conducted using Cell Proliferation ELISA, BrdU kit (Roche) as described previously [15].

2.7. Transfection of target-specific siRNAs for HIF-1α or EGFR knockdown

Potential small interfering RNA target sites for mRNA sequences of porcine HIF-1α or EGFR were determined using the Invitrogen design program as described previously [15].

2.8. Statistical analyses

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

3. Results

3.1. Accumulation of HIF-1 proteins in an oxygen-dependent manner in pTr cells

To determine whether pTr cells activate the HIF system in response to oxygen deficiency, we measured expression of HIF-1 mRNAs and proteins in pTr cells cultured under normoxic (20% O_2) or hypoxic (5% O_2) conditions for 16 h. As shown in Fig. 1A, expression of *HIF-1α* mRNA or *HIF-1β* mRNAs was not affected ($P > 0.05$) by concentration of oxygen. However, western blotting showed that hypoxia resulted in a 13.7-fold increase ($P < 0.001$) in HIF-1α protein in pTr cells, compared to values for pTr cells cultured under normoxic conditions (Fig. 1B). On the other hand, there was no effect ($P > 0.05$) of oxygen status on HIF-1β protein in the pTr cells. HIF-1α protein was detected at very low levels in nuclei and cytoplasm in pTr cells under 20% oxygen, but pTr cells cultured under hypoxic conditions exhibited abundant amounts of immunoreactive HIF-1α protein especially in the nucleus (Fig. 1C). On the other hand, HIF-1β protein was abundant in pTr cells, irrespective of oxygen concentration. These results indicate that changes in abundance of HIF-1α protein is likely to be principally involved in the metabolic adaptation of pTr cells to changes in oxygen concentrations.

3.2. Stimulation of HIF-1α accumulation in response to EGF through transcriptional and posttranscriptional regulation

We next examined whether the abundance of HIF-1α protein