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Exposure of decidualized HIESC to low oxygen tension and leucine deprivation results in increased IGFBP-1 phosphorylation and reduced IGF-I bioactivity

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ABSTRACT

Phosphorylation of decidual IGFBP-1 enhances binding of IGF-I, limiting the bioavailability of this growth factor which may contribute to reduced placental and fetal growth. The mechanisms regulating decidual IGFBP-1 phosphorylation are incompletely understood. Using decidualized human immortalized endometrial stromal cells we tested the hypothesis that low oxygen tension or reduced leucine availability, believed to be common in placental insufficiency, increase the phosphorylation of decidual IGFBP-1. Multiple reaction monitoring-MS (MRM-MS) was used to quantify IGFBP-1 phosphorylation. MRM-MS validated the novel phosphorylation of IGFBP-1 at Ser58, however this site was unaffected by low oxygen tension/leucine deprivation. In contrast, significantly elevated phosphorylation was detected for pSer119, pSer98/pSer101 and pSer169/pSer174 sites. Immunoblotting and dual-immunofluorescence using phosphosite-specific IGFBP-1 antibodies further demonstrated increased IGFBP-1 phosphorylation in HIESC under both treatments which concomitantly reduced IGF-I bioactivity. These data support the hypothesis that down regulation of IGF-I signaling links decidual IGFBP-1 hyperphosphorylation to restricted fetal growth in placental insufficiency.

1. Introduction

Fetal growth restriction (FGR) increases the risk of perinatal complications and is linked to the development of obesity, diabetes and cardiovascular disease later in life (Gluckman et al., 2008). However, the pathophysiology underlying FGR is not yet clearly understood. The insulin-like growth factor (IGF) system is a critical regulator of fetal growth (Baker et al., 1993). Short term maternal undernutrition in animal models leads to cessation of fetal growth associated with reduced fetal IGF-I levels and altered circulating concentrations of IGF binding proteins (IGFBPs) (Gluckman and Pinal, 2003; Baxter, 2000). The activity of IGFs is strongly modulated by IGFBPs. Because binding of IGFs to IGFBPs prevents association of the growth factor to its receptors, IGFBPs can inhibit the stimulatory actions of IGFs on cellular proliferation, migration and differentiation (Baxter, 2000). Conversely, binding of IGFBPs to IGFs can in some situations have stimulatory effects, possibly by bringing the growth factor in close proximity to the receptor, therefore augmenting the interaction (Baxter, 2000).

Overexpression of hepatic IGFBP-1 in transgenic mice has been shown to cause a 15–20% reduction in birth weight (Watson et al., 2006), providing strong evidence that hepatic IGFBP-1 is an important endocrine regulator of fetal growth. Low oxygen tension (hypoxia) strongly induces IGFBP-1 protein and mRNA expression levels which plays an important role in regulating embryonic/fetal growth (Kajimura et al., 2005). A role for hypoxia-inducible factor 1 (HIF-1) and hypoxia-response element 1 (HRE1) in induction of IGFBP-1 gene expression has been proposed previously to restrict

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IGF-mediated fetal growth (Tazuke et al., 1998). IGFBP-1 is elevated in fetuses with long-term, chronic hypoxia. Low oxygen tension triggers IGFBP-1 secretion also in hepatocellular carcinoma HepG2 cells (Tazuke et al., 1998) as well as in primary hepatocytes isolated from human fetuses (Popovici et al., 2001). In addition, reduced amino acid availability also increases the expression of IGFBP-1 (Averous et al., 2005). These findings implicate stress-responsive alterations in fetal IGFBP-1/IGF-I system to play an important role in the development of FGR.

Central to IGF-I bioactivity in FGR is the phosphorylation status (sites and degree) of IGFBP-1. Given that the affinity of phosphorylated IGFBP-1 for IGF-I is greater than that of non-phosphorylated IGFBP-1, phosphorylated IGFBP-1 typically inhibits the IGF-I/IGF-1R interaction (Jones et al., 1993a). We have previously demonstrated that IGFBP-1 phosphorylation in HepG2 cells is induced in response to both leucine deprivation and hypoxia and increases its affinity for IGF-I up to 300-fold (Seferovic et al., 2009). This increase in IGF-I affinity results in a pronounced inhibition of cellular proliferation and inhibition of IGF-I bioactivity during periods of cellular stress (Seferovic et al., 2009; Damerill et al., 2016); these data are consistent with several literature reports showing that phosphorylation of IGFBP-1 more potently inhibits IGF-I stimulated cell proliferation, DNA synthesis, amino acid transport and apoptosis (Firth and Baxter, 2002). We have also previously reported that IGFBP-1 phosphorylation is markedly increased at three specific serine residues (Ser101, Ser119 and Ser169) in the amniotic fluid of human growth restricted fetuses (Abu Shehab et al., 2010). We also have shown that IGFBP-1 phosphorylation is increased in the fetal liver of FGR baboons (Abu Shehab et al., 2014). Together these data indicate that changes in the phosphorylation status of fetal IGFBP-1 may directly contribute to abnormal fetal growth.

During implantation and early pregnancy ovarian hormones promote the morphological and functional transformation of endometrial stromal cells into decidua, which play an important role in regulating trophoblast invasion. The decidua is also the major source of maternal circulating IGFBP-1 (Martina et al., 1997; Gibson et al., 2001) and the decidua therefore controls the bioavailability of maternal IGF-I. Because maternal IGF-I is an important regulator of placental growth and function, including placental nutrient transport (Chellakooty et al., 2004), the decidua plays a critical role in modulating maternal-fetal resource allocation (Martina et al., 1997). A lack of normal increase in uteroplacental blood flow results in placental insufficiency, which is a common cause of FGR. Placental insufficiency is believed to be associated with placental hypoxia and decreased levels of nutrients (Hutter et al., 2010). It is therefore possible that increased phosphorylation of decidual IGFBP-1 by hypoxia and nutrient deprivation may contribute to the restricted fetal growth indirectly by decreasing the bioavailability of IGF-I in the maternal circulation.

The gestational age related changes in phosphoisoform patterns in decidual explants have been reported previously (Martina et al., 1997), however, the status of IGFBP-1 phosphorylation at specific sites is currently unknown and there are no previous reports of mechanisms regulating decidual IGFBP-1 phosphorylation in normal pregnancy or in association to placental insufficiency. We tested the hypothesis that decreased oxygen or leucine availability, believed to be common in placental insufficiency, increase the phosphorylation of decidual IGFBP-1. We used decidualized human immortalized endometrial stromal cell (HIESC) (Chapdelaine et al., 2006) as a model system for determining the effects of hypoxia and leucine deprivation on decidual IGFBP-I phosphorylation. Multiple reaction monitoring mass spectrometry (MRM-MS) was applied as a targeted approach (Damerill et al., 2016) to quantify the site-specific IGFBP-1 phosphorylation in decidualized HIESC. Our extensively validated phosphosite-specific IGFBP-1 antibodies (Abu Shehab et al., 2014; Abu Shehab et al., 2013) were utilized for immunoblot analysis as well as subcellular localization using dual immunofluorescence immunocytochemistry (ICC). Moreover, inhibitory effects of IGFBP-1 hyperphosphorylation on IGF-I bioactivity were assessed using IGF-1R autophosphorylation (Abu Shehab et al., 2013). We further tested the impact of changes in IGFBP-1 phosphorylation on IGF-I signaling activity to establish the biological/physiological relevance of the overall findings in this study.

2. Materials and methods

2.1. Cell culture and in vitro decidualization

HIESC (a gift, Dr. M Fortier, U Laval, Canada) were plated in 12 well plates at 75% confluence and cultured in RPMI 1640 medium without phenol red (Life Technologies, Burlington, ON) enriched with 10% fetal bovine serum (FBS) and 50 IU penicillin-streptomycin (Life Technologies, Burlington, ON) at 37 °C with 5% CO_2. After 24 h, the cell monolayer was washed in phosphate-buffered saline (PBS). For decidualization, media was replaced with RPMI 1640 media but containing 2% dialyzed FBS (stripped using Dextran Coated Charcoal (DCC; Sigma Aldrich, ST Louis MO), 0.5 mM 8-bromo cAMP (Cayman Chemicals, Ann Arbor, MI) and 1.0 μM medroxyprogesterone acetate (MPA) (Pfizer Canada, Kirkland, Quebec). The media was changed every 48 h. After six days of decidualization, the media was collected for the assessment of IGFBP-1 secretion and phosphorylation, whereas cells were lysed in lysis buffer (Cell Signaling Technologies, Danvers, MA) and studied as described below.

2.2. Changes in morphology following decidualization of HIESC by shape index calculation

The morphological characteristic of HIESC was determined before and after decidualization using the approach of calculating the shape index as described (Kajihara et al., 2014). Phase contrast images were captured 6 days after decidualization (treatment with cAMP + MPA). Individual cells (50 cells) were outlined from three cultures (n = 3) for control and treated cells and the roundness factor was determined using Image Pro Premier software. The shape index formula used was as reported by Kajihara et al. (2014) (4pi x area/perimeter^2). A circular object would have a shape index of 1 whereas a straight line has an index of 0.

2.3. Decidualized HIESC culture in low-oxygen tension

After six days of decidualization, HIESC were exposed to low-oxygen tension (1% O_2). The cells were cultured in decidualization media and then placed either in incubator air (normoxia) or a sealed hypoxia chamber, filled with a 1% O_2, 5% CO_2, balanced N_2 gas mixture (Praxair Canada Ltd) for 15 min to ensure saturation. Thereafter, the sealed chamber was placed in a tissue culture incubator at 37 °C on an orbital shaker for 24 h. Based on our previous studies (Seferovic et al., 2009, 2011; Damerill et al., 2016; Seferovic and Gupta, 2016) utilizing a Hudson 5590 Oxygen Monitor (Hudson, Ventronics Division) the saturation of the O_2 in the chamber has been shown to remain consistent for up to 72 h. After treatment, the cell media and cell lysate were collected and stored at −80 °C for further analysis.

2.4. Leucine deprivation of decidualized HIESC

Cells were cultured in 12 well plates in decidualization media for six days. Subsequently, prior to harvesting, the cells were