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IGFBP-1 hyperphosphorylation in response to leucine deprivation is mediated by the AAR pathway

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ABSTRACT

Insulin-like growth factor-1 (IGF-I) is the key regulator of fetal growth. IGF-I bioavailability is markedly diminished by IGF binding protein-1 (IGFBP-1) phosphorylation. Leucine deprivation strongly induces IGFBP-1 hyperphosphorylation, and plays an important role in fetal growth restriction (FGR). FGR is characterized by decreased amino acid availability, which activates the amino acid response (AAR) and inhibits the mechanistic target of rapamycin (mTOR) pathway. We investigated the role of AAR and mTOR in mediating IGFBP-1 secretion and phosphorylation in HepG2 cells in leucine deprivation. mTOR inhibition (rapamycin or raptor + rictor siRNA), or activation (DEPTOR siRNA) demonstrated a role of mTOR in leucine deprivation-induced IGFBP-1 secretion but not phosphorylation. When the AAR was blocked (U0126, or ERK/GCN2 siRNA), both IGFBP-1 secretion and phosphorylation (pSer101/pSer119/pSer169) due to leucine deprivation were prevented. CK2 inhibition by TBB also attenuated IGFBP-1 phosphorylation in leucine deprivation. These results suggest that the AAR and mTOR independently regulate IGFBP-1 secretion and phosphorylation in response to decreased amino acid availability.

1. Introduction

Although the etiology of fetal growth restriction (FGR) is multifactorial, this condition is often a result of placental insufficiency, i.e. the inability of the placenta to effectively deliver nutrients and oxygen to the fetus (Brown et al., 2011; Miller et al., 2008). FGR infants fail to achieve their full growth potential after suffering nutritional deprivation (Jang et al., 2011) and are at an increased risk for perinatal mortality (Pallotto and Kilbride, 2006). FGR affects ~5 to 7% of all pregnancies (Romo et al., 2009) and FGR babies are predisposed to greater risks of childhood and adult metabolic, cardiovascular, and neurological complications (Barker, 2006; Baschat, 2004; Pallotto and Kilbride, 2006). The mechanisms linking decreased nutrient availability and reduced fetal growth are not well understood.

Insulin-like growth factor I (IGF-I), synthesized mainly by the fetal liver, is a key regulator of fetal growth. Fetal serum IGF-I levels are significantly reduced in the growth-restricted fetuses (Leger et al., 1996). IGF binding protein-1 (IGFBP-1) is primarily secreted by the fetal liver (Lee et al., 1993) and constitutes the key fetal circulating IGFBP (Han et al., 1996). Elevated fetal circulating IGFBP-1 and decreased IGF-I levels are strongly correlated with FGR onset (Larsson et al., 2013; Reece et al., 1994; Watson et al., 2006). IGFBP-1 functions by binding IGF-I and sequestering it from its receptor, IGF-1R, consequently preventing it from transducing mitogenic signals (Clemmons et al., 1995; Firth and Baxter, 2002; Valentinis and Baserga, 2001). Phosphorylation of IGFBP-1 increases the binding affinity of IGFBP-1 for IGF-I (Jones et al., 1991) and sequesters IGF-I, thereby decreasing IGF-I bioavailability (Firth and Baxter, 2002; Koistinen et al., 1990; Wong et al., 1999; Yu et al., 1998).

Human FGR fetuses often have decreased fetal circulating levels of essential amino acids, such as leucine (Regnault et al., 2013; Sathishkumar et al., 2011; Teodoro et al., 2012; Wu, 2009). In our laboratory, we have previously demonstrated that leucine deprivation induces hyperphosphorylation of IGFBP-1 in HepG2 cells at discrete sites, which markedly increased the affinity of IGFBP-1 for IGF-I, and inhibited IGF-I-dependent cell growth (Seferovic et al., 2009). Although modest increases in IGFBP-1 phosphorylation were found in HepG2 cells cultured under lower leucine concentrations (70 and 140 μM leucine), leucine deprivation (0 μM leucine) distinctly increased IGFBP-1 phosphorylation compared to HepG2 cells cultured with leucine (450 μM leucine) (Seferovic et al., 2009).

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Furthermore, we have recently demonstrated that IGFBP-1 phosphorylation are increased at three different sites (Ser101, Ser119, and Ser169) in human umbilical cord plasma from FGR pregnancies and in liver from baboon FGR fetuses (Abu Shehab et al., 2014). These data indicate that increased phosphorylation of IGFBP-1 at specific sites plays an important role in FGR pathogenesis.

FGR is characterized by decreased amino acid availability (Maulik, 2006; Miller et al., 2008), which activates the amino acid response (AAR) (Strakovsky et al., 2010) and inhibits mechanistic target of rapamycin (mTOR) signaling (Roos et al., 2007, 2009). Amino acids, oxygen and growth factor signaling activate mTOR signaling (Arsham et al., 2003; Martin and Sutherland, 2001; Roos et al., 2009; Wullschleger et al., 2006). mTOR integrates nutrient and mitogenic signals to regulate cell growth and cell division (Wang and Proud, 2011). mTOR exists in two complexes, mTOR Complex 1 (mTORC1) and 2, with the protein raptor associated to mTORC1 and rictor associated to mTORC2 (Oshiro et al., 2004; Sarbassov et al., 2004). Activated mTORC1 phosphorylates 4E-BP1 and p70-S6K and promotes protein translation (Foster and Fingar, 2010; Wang and Proud, 2011). mTORC2 phosphorylates Akt and PKCα and regulates cell metabolism and survival (Foster and Fingar, 2010; Sarbassov et al., 2004). Oxygen, growth factor and amino acids, particularly leucine and arginine, activate mTORC1 signaling (Chotechuang et al., 2009; Hara et al., 1998; Li et al., 2011; Matsumura et al., 2005; Wullschleger et al., 2006). mTORC1, in particular, is inhibited by the binding of rapamycin although longer treatments and higher doses of rapamycin have also been shown to inhibit mTORC2 (Abu Shehab et al., 2014; Foster and Fingar, 2010).

We have previously shown using HepG2 cells and baboon fetal hepatocytes in vitro that inhibition of mTOR signaling resulted in increased IGFBP-1 phosphorylation at the three specific sites (Abu Shehab et al., 2014). In addition using a baboon model of FGR in the same study we also demonstrated that increased site-specific IGFBP-1 phosphorylation in FGR is linked to an inhibition of the mTOR and stimulation of protein kinase CK2. However, the mechanisms underlying IGFBP-1 hyperphosphorylation specifically in conditions of amino acid deprivation remain to be established.

The AAR pathway is activated under conditions of cellular nutrient stress (Deng et al., 2002; Dong et al., 2000; Hinnebusch, 1997; Kilberg et al., 2005; Strakovsky et al., 2010; Thiaville et al., 2008; Zhou and Pan, 2011). General control non-derepressible 2 (GCN2) is the key sensor of cellular nutrient status, which is activated upon sensing excess uncharged cytoplasmic tRNAs (Dong et al., 2000; Hinnebusch, 1997). Leucine deprivation activates and phosphorylates GCN2 at pThr898, which subsequently phosphorylates eukaryotic initiation factor 2 (eiF2) at pSer51 of the alpha subunit (eiF2α) (Deng et al., 2002). Phosphorylation of eiF2α (pSer51), which is increased in FGR (Yung et al., 2008), proceeds to inhibit eIF2B activity and therefore overall global protein synthesis while concurrently promoting the translation of certain stress-responsive mRNAs, including activating transcription factor 4 (ATF4) (Deng et al., 2002; Hinnebusch, 1997). ATF4 is a critical stress-responsive transcription factor, which, when synthesized, promotes the transcription of several growth-arresting genes (Kilberg et al., 2009). eiF2α (pSer51) phosphorylation and total ATF4 expression levels are therefore functional readouts of AAR activity. The role of the AAR signal transduction pathway in regulating IGFBP-1 phosphorylation has, to our knowledge, not previously been investigated.

In this study, we hypothesized that inhibition of mTOR signaling and AAR activation increase IGFBP-1 secretion and phosphorylation at specific sites in response to amino acid deprivation. We used HepG2 cells as a model for human fetal hepatocytes (Hart et al., 2010; Kelly and Darlington, 1989; Maruyama et al., 2007; Pal et al., 2012; Wilkening et al., 2003) to investigate the mechanisms linking mTOR and AAR signaling with IGFBP-1 phosphorylation under leucine deprivation. We studied the secretion and phosphorylation of IGFBP-1 in HepG2 cells in response to mTOR inhibition (rapamycin) or AAR inhibition (U0126). Alternatively, cells were transfected with siRNA targeting raptor + rictor or DEP domain-containing mTOR-interacting protein (DEPTOR) (to inhibit or activate mTORC1 and C2, respectively) (Foster and Fingar, 2010), and ERK1/2 and/or GCN2 (to inhibit ERK-mediated AAR) (Hinnebusch, 1997; Thiaville et al., 2008) in cells cultured with or without leucine. Finally, we confirmed that changes in IGFBP-1 phosphorylation under leucine deprivation altered IGF-I bioactivity by employing our previously established IGF-1R autophosphorylation assay (Abu Shehab et al., 2013, 2014) which supported the functional significance of our findings.

2. Methods

2.1. Cell culture

Human hepatocellular carcinoma cells (HepG2), purchased from ATCC (Mananassas, VA), were cultured in Dulbecco's modified Eagle medium with nutrient mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA) at 37 °C in 20% O_2 and 5% CO_2 as we described previously (Abu Shehab et al., 2014; Seferovic et al., 2009).

2.2. Leucine deprivation

HepG2 cells were treated in specialized DMEM/F12 selectively deprived and restored of specific amino acids and were incubated in the specialized media either deprived of (0 μM) or supplemented with (450 μM) leucine as we described in our previous study (Seferovic et al., 2009).

Cells were further incubated in leucine containing or leucine deprived media during rapamycin (100 nM), U0126 (10 μM), or TBB (1 μM) treatments or following transfection with siRNA. Cell media and cell lysate were collected following 24 hour (chemical treatments) or 72 hour (siRNA treatments) exposure to the specialized media.

2.3. Inhibitor treatments

HepG2 cells were plated in 12-well culture dishes until cultures reached 75% confluence then starved for 6 hours in 2% FBS (DMEM/F12) prior to treatments with pharmacological inhibitors. Based on previous dose-dependency data, HepG2 cells were treated post-6 hour starvation for 24 hours using 100 nM rapamycin, 1 μM TBB as we reported previously (Abu Shehab et al., 2014) or treated with 10 μM U0126 after assessment via dose-dependency experiments (data not shown). Following treatments, cell media and cell lysate were prepared as we described (Abu Shehab et al., 2014) and stored at –80 °C.

2.4. RNA interference silencing

HepG2 cells were plated at 65% confluence in 12-well culture plates. Silencing using siRNA targeting raptor + rictor, DEPTOR, GCN2 (Sigma-Aldrich, St. Louis, MO, USA) or ERK (Cell Signaling Technologies, Beverly, MA, USA) in HepG2 cells was achieved using transfection with 100 nM siRNA and 5 μL Dharmafect transfection reagent 4 (Thermo Scientific, Rockford, IL, USA) in regular, serum free DMEM/F12. To simultaneously ensure maximal silencing and maximize cell survival, the transfected cell medium was replaced after 24 hours with specialized leucine containing or leucine deprived medium and studied after 72 hours (96 hours following

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