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Placental Glycogen Stores are Increased in Mice with *H19* Null Mutations but not in those with Insulin or IGF Type 1 Receptor Mutations

Diego R. Esquiliano, Weihui Guo, Li Liang, Pieter Dikkes, Mary Frances Lopez*

Endocrine Division, Children's Hospital and Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

The function of glycogen in the placenta remains controversial. Whether it is used as a source of fuel for placental consumption or by the fetus in times of need has yet to be determined. Two imprinted genes, insulin-like growth factor 2 (*Igf2*) and *H19* are highly expressed in the placenta. We have previously demonstrated that mice with *Igf2* deficiency have lower levels of placental glycogen. In this study, we used mice with targeted disruption of the *H19* gene ($H19^{-/-}$) to determine the importance of Igf2 overexpression in placental growth and glycogen stores. In addition, since *Igf2* mediates most of its functions by signaling through the insulin and/or IGF Type 1 receptors, we determined whether gene deletions to these receptors could affect placental glycogen stores. Our data demonstrate that placentas from $H19^{-/-}$ mice are heavier, have higher number of glycogen cells, and contain larger glycogen concentrations than those of $H19^{+/+}$ mice. No differences in GSK-3, ERK, or total Akt expression or phosphorylation were found between genotypes; however, Akt1 protein expression levels were significantly increased in $H19^{-/-}$ placentas. Results obtained from insulin receptor or IGF Type 1 receptor mutant mice did not show differences in placental glycogen content compared to their wild-type littermates, supporting the notion of a specific placental Igf2 receptor. Taken together, these results support a role for Igf2 and Akt1, but not the insulin nor the IGF Type 1 receptors, in the regulation of placental growth and glycogen metabolism.

1. Introduction

One crucial function of the placenta is to transfer glucose from the maternal circulation into the fetus [1]. This glucose is used as a source of energy by the placenta and the fetus or stored as glycogen [1]. While the glycogen in the fetal liver is used during the neonatal period to maintain levels of glycemia, the role of glycogen in the placenta remains to be fully determined. Some studies support the idea that placental glycogen serves as a source of energy for its own needs [2], while others have raised the possibility that it is also available for fetal needs [3–5]. In their studies, Barash and collaborators demonstrated that placental glycogen could be mobilized in response to hormonal stimuli; however, they postulated that glucose could not be produced in the placenta due to the absence of glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis [6]. Recent studies, however, have demonstrated the existence of a specific G6Pase gene present in the placenta [5]. This gene is unrelated to the hepatic G6Pase gene, thus suggesting that the placenta could generate glucose that potentially play a role in maintaining fetal blood glucose concentrations [5]. The placenta accumulates high glycogen concentrations during early gestation but these levels decrease near term [1]. This drop in glycogen is thought to be caused by the fetus placing high demands on maternal resources needed for growth during the final stages of gestation and thus using available placental glycogen reserves to meet those demands [7].

Although insulin plays an important role in postnatal glycogen metabolism [8], this hormone does not appear to play a role in placental glycogen metabolism [9]. Insulin promotes glycogen synthesis by increasing the activity of glycogen synthase and decreasing the activity of glycogen phosphorylase [8]. These two enzymes are present in the placenta but do not seem to be affected by insulin [9]. Insulin-like growth factor II (Igf2) is a hormone that is highly expressed in both the placenta and fetus [10,11]. It plays an important role in placental growth [12,13], nutrient transfer [14], and glycogen synthesis [13]. Placentas from Igf2 knockout ($Igf2^{-/-}$)

* Corresponding author. Endocrine Division, Children's Hospital and Harvard Medical School, 300 Longwood Ave., Karp #4212, Boston, MA 02115, USA. Tel.: +1 617 919 2862; fax: +1 617 730 0244.
E-mail address: mary.lopez@childrens.harvard.edu (M.F. Lopez).

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mice are significantly smaller than those of their wild-type ($Igf2^{+/+}$) littermates [12,13], have disproportionately fewer numbers of glycogen cells, and have lower glycogen concentrations than those of $Igf2^{+/+}$ mice, [13], thus supporting the role of Igf2 in placental glycogen metabolism.

Another gene involved in fetal and placental growth is *H19* [15,16]. *H19* is an unusual gene that does not encode a protein. H19 is abundantly expressed in the developing embryo and the placenta [15,17,18]. Both, *Igf2* and *H19* are present within the same chromosome, only 90 Kb apart, and are part of a cluster of genes that are imprinted. They have similar expression in the fetus but they are reciprocally imprinted; while *Igf2* is paternally transcribed, *H19* is maternally expressed [19,20]. Mice with targeted deletion of the *H19* gene ($H19^{-/-}$) have increased Igf2 expression and are born 30% larger than their wild-type ($H19^{+/+}$) littermates [15]. The increased size is thought to be due to increased Igf2 expression rather than a loss of H19 function. The maternal Igf2 allele, which is usually silent, becomes transcriptionally active leading to an increase in Igf2 mRNA expression from both paternal alleles and consequently to fetal and placental overgrowth [15,16].

Insulin-like growth factor 2 binds to three receptors: the IGF type 1 receptor (Igf1R), the IGF type 2 receptor (Igf2R), and the insulin receptor (InsR) [21,22]. These receptors may compete for Igf2 binding during placental development since they are all expressed in the placenta. The Igf1 and Igf2 receptors are abundantly expressed in the labyrinth zone of the placenta and are present in small amounts in the basal zone [11]. The InsR is expressed in both the labyrinth and basal zones [23]. The InsR and Igf1R are distinct, but related receptors that can mediate responses to any of the three ligands [24]. In addition to these three receptors, Efstratiadis and collaborators have postulated an additional placental receptor, which they named XR_p [25]. This receptor is thought to mediate some, if not all of the functions of Igf2 in the placenta [25].

In summary, the present study shows that Igf2 over-expression in $H19^{-/-}$ placentas results in higher levels of placental glycogen stores. Since Igf2 binds to the Igf1R and InsR, we also measured glycogen levels in placentas of *Igf1R* and *InsR* deficient mice to determine the importance of these receptors in placental glycogen regulation. In addition, we also began to elucidate potential mechanisms that may be involved in regulating the over-growth phenotype observed in the $H19^{-/-}$ placentas. Understanding placental glycogen metabolism will help us to better understand carbohydrate metabolism during gestation and its effects on fetal growth.

2. Materials and methods

2.1. Animals and tissue collection

Mice carrying the inactivated *H19*, *Igf1R*, and *InsR* genes [15,25,26] were maintained as separate colonies. Knockout (KO) and wild-type (WT) mice from each mutation were derived from the same progenitors, using heterozygous matings. Mice were kept on a 12 h light, 12 h dark schedule (lights on at 7:00 a.m.) and allowed free access to food and water. Eight to 10 week old females were housed with adult male mice and examined daily for vaginal plugs. The presence of a vaginal plug on the morning after introduction of the female into the male cage was designated as day 0 of pregnancy. Placentas were collected from mice on days 15 and 18 of gestation for morphological analysis and determination of glycogen levels. Experiments were performed using at least three litters per genotype and were limited to litters containing 7–10 conceptuses and their respective placentas. The mice were housed and cared for according to National Institutes of Health guidelines and all experiments were approved by the Animal Care and Use Committee of Children's Hospital, Boston.

2.2. Mice genotyping

The genotype of $H19^{-/-}$ and $Igf2^{-/-}$ mice, and their respective wild-type (WT) littermates was determined by Southern blot analysis as described previously [12,15], whereas the genotype of mice carrying the *Igf1R* or *InsR* deletions were genotyped by PCR analysis [25,26].

2.3. Histological analysis

Placentas were fixed by immersion in either Carnoy's (for H&E staining) or Rossman's (for Best's carmine staining) fixative for 24 h. All tissues used in this study were embedded in paraffin using an automated tissue processor. Serial 5 μm sections were cut using a Reichert-Jung Biocut rotatory microtome. Prior to staining, paraffin was removed from the sections with three changes of xylene, followed by rehydration in descending concentrations of ethanol (100%, 90%, 70%, 50%) and water. Sections were stained with hematoxylin–eosin for morphological analysis or with Best's carmine for glycogen content [27]. Diastase treatment was used before or after Best's carmine staining to show that staining was specific to glycogen.

2.4. Counting of placental cells

Cells were counted in representative sections using an ocular grid in a Zeiss Axioskop as described previously [13]. Micrographs of three sections of each placenta were used to count and compare cell number in morphologically equivalent areas of $H19^{+/+}$ and $H19^{-/-}$ placentas. The most lateral section, that contained both the basal and labyrinth zones, was chosen as the first section. The second section was halfway to the midline of the placenta. The third section was at the middle of the placenta. Three sections from each of three different placentas harvested from different litters were used for the analysis of each phenotype [13].

2.5. Glycogen assay

Glycogen in placental tissues was measured as described previously [28]. Briefly, placentas were digested with 30% KOH saturated with Na_2SO_4 at 95 °C for 30 min.

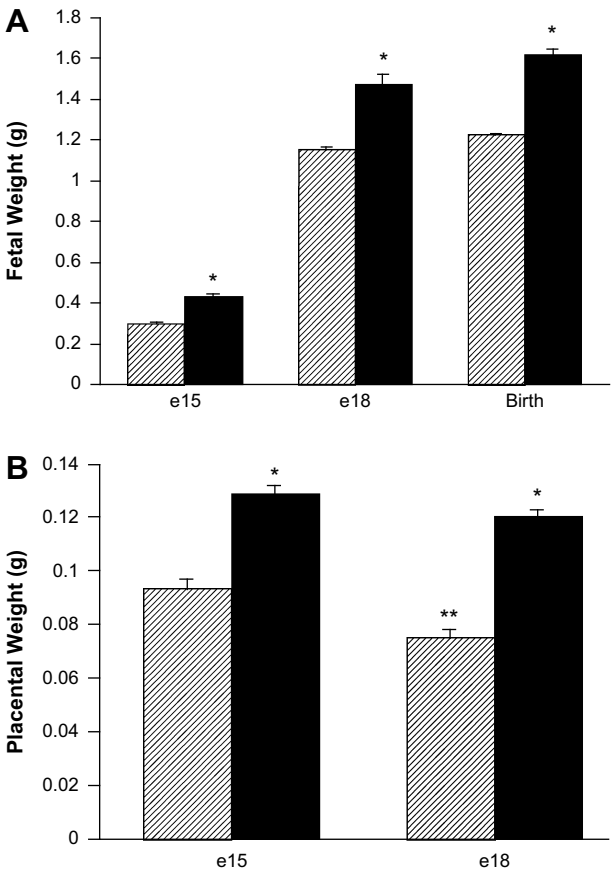

Fig. 1. *H19* deficiency affects fetal (A) and placental weight (B). The mean values are shown in solid bars for the $H19^{-/-}$ mice and hatched bars for $H19^{+/+}$ mice. $H19^{-/-}$ fetuses weighed significantly more on embryonic days 15 ($n = 9$) and 18 ($n = 19$), and at birth ($n = 34$) compared to $H19^{+/+}$ fetuses ($n = 15, 11, 47$, respectively). $H19^{-/-}$ placentas also weighed significantly more on embryonic days 15 ($n = 24$) and 18 ($n = 12$) compared to $H19^{+/+}$ controls ($n = 15$ and 12, respectively). Data are mean ± SEM. $^{*}P < 0.01$. Notice that gestational decrease in weight occurred in $H19^{+/+}$ but not in $H19^{-/-}$ placentas on day 18 of gestation $^{**}P < 0.01$.