PDM*CE*

Programa **D**outoral em **M**etabolismo:
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U.PORTO
FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

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Impact of Gestational Diabetes Mellitus in the maternal-to-fetal transport of nutrients and on placental development

Porto, 2013

DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR EM METABOLISMO - CLÍNICA E EXPERIMENTAÇÃO APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

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Artigo 48º § 3º - A Faculdade não responde pelas doutrinas expedidas na dissertação (Regulamento da Faculdade de Medicina do Porto – Decreto-Lei nº 19337, 29 de Janeiro de 1931).

O trabalho experimental foi financiado pela Fundação para a Ciência e Tecnologia (Projecto: PTDC/SAU-OSM/102239/2008). O candidato realizou trabalho experimental com o apoio de uma bolsa de Doutoramento (SFRH/BD/63086/2009) atribuída pela Fundação para a Ciência e Tecnologia.

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CONTRIBUTO PESSOAL NOS TRABALHOS DE INVESTIGAÇÃO APRESENTADOS NESTA DISSERTAÇÃO

A realização experimental foi executada no Departamento de Bioquímica da Faculdade de Medicina da Universidade do Porto. Declaro que tive uma contribuição determinante em toda a realização experimental do trabalho (programação e execução das experiências), bem como na interpretação e discussão dos resultados apresentados em todos os artigos desta dissertação. Declaro ainda que colaborei ativamente na redação dos artigos.

ACKNOWLEDGEMENTS

Às Professoras Fátima Martel e Elisa Keating (Departamento de Bioquímica da FMUP), por toda a orientação científica, transmissão de conhecimentos, paciência, simpatia, motivação e exemplo pessoal, um profundo obrigado.

Aos colaboradores mais diretos, Ana Branco, Cláudia Pereira (Departamento de Bioquímica da FMUP), Professora Carla Ramalho, Tiago Ferraz (Departamento de Ginecologia e Obstetrícia do Centro Hospitalar de São João) e Maria João Pinho (Departamento de Farmacologia da FMUP), pela ajuda e disponibilidade, um sincero obrigado.

Aos meus amigos e colegas do Departamento de Bioquímica da FMUP, Ana Faria, Ana Pirraco, Ângela, Carla Costa, Cidália, Claúdia Azevedo, Cláudia Marques, Diana, Diogo, Delfim, Emanuel, Manuela, Marta, Marisa, Susana, Pedro, Raquel Costa e Sónia, por todo o companheirismo, simpatia e apoio científico e laboratorial, um sincero obrigado.

Às Professoras e Professores do Departamento de Bioquímica da FMUP, Alejandro Santos, Conceição Calhau, Duarte Torres, Hipólito-Reis, Isabel Azevedo, João Tiago Guimarães, Laura Ribeiro, Maria João Martins, Nuno Alçada, Rita Negrão, Rosário Monteiro e Rui Fontes, pela sensibilização do ensino da Bioquímica, por darem-me a possibilidade de participar em diferentes projetos de investigação, pelos conselhos, e pela amizade, um agradecimento sincero.

À Professora Raquel Soares, diretora do Programa Doutoral em Metabolismo – Clínica e Experimentação e do Departamento Bioquímica da FMUP pelo exemplo de dedicação à formação científica e pela disponibilidade e amizade, um agradecimento sincero.

À minha família, pelo apoio perene e incondicional, o mais profundo dos agradecimentos.

ABBREVIATIONS

AA: arachidonic acid

ACSL: long-chain acyl-CoA synthetases

ATP: adenosine-5'-triphosphate

BM: basal membrane

CAT: cationic amino acid transporters

DG: 2-deoxy-D-glucose

DHA: docosahexaenoic acid

DNA: deoxyribonucleic acid

DTB cells: human cytotrophoblasts obtained from GDM pregnancies

EFSA: European Food Safety Authority

EGCG: epigallocatechin-3-gallate

ERK: extracellular-signal-regulated-kinase

FA: folic acid

FABP: fatty acid binding proteins

FAT/CD36: fatty acid translocase

FATP: fatty acid transport proteins

FGR: fetal growth restriction

FRα: folate receptor alpha

GDM: gestational diabetes mellitus

GLUT: facilitative glucose transporters

GPx: glutathione peroxidase

GSH: reduced glutathione

GSSG: oxidized glutathione

HAPO: hyperglycemia and adverse pregnancy outcomes

IADPSG: International Association of Diabetes in Pregnancy Study Group

IL: interleukin

JAK/STAT: janus kinases/signal transducers and activators of transcription

JNK: c-Jun-N-terminal kinase

L-Ala: L-Alanine

LAT: L-type amino acid transporter

LC-PUFAs: long-chain polyunsaturated fatty acids

LGA: large-for-gestational age

L-Lys: L-Lysine

L-Met: L-Methionine

L-Ser: L-Serine

LXR: liver X receptors

MAPK: mitogen-activated protein kinases

MDA: malonaldehyde

MVM: microvillous membrane

NEM: *N*-ethylmaleimide

NTB cells: human cytotrophoblasts obtained from normal pregnancies

NTDs: neural tube defects

OGTT: oral glucose tolerance test

PCFT: proton-coupled folate transporter

pFABPpm: placental plasma membrane fatty acid binding protein

PI3K: phosphatidylinositol 3-kinase

PKA: protein kinase A

PKC: protein kinase C

RFC1: reduced folate carrier

RNA: ribonucleic acid

ROS: reactive oxygen species

SNAT: sodium-coupled neutral amino acid transporter

STB: syncytiotrophoblast

T1D: type 1 diabetes

TBHP*: tert*-butylhydroperoxide

TNF-α: tumor necrosis factor-alpha

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

A. Araújo JR, Correia-Branco A, Moreira L, Ramalho C, Martel F, Keating E. Folic acid uptake by the human syncytiotrophoblast is affected by gestational diabetes, hyperleptinemia, and TNF-α. Pediatr Res. 2013;73:388-394.

B. Araújo JR, Correia-Branco A, Ramalho C, Gonçalves P, Pinho MJ, Keating E, Martel F. L-methionine placental uptake: characterization and modulation in gestational diabetes mellitus. Reprod Sci. 2013;20:1492-1507.

C. Araújo JR, Correia-Branco A, Ramalho C, Keating E, Martel F. Gestational diabetes mellitus decreases placental uptake of long-chain polyunsaturated fatty acids: involvement of long-chain acyl-CoA synthetase. J Nutr Biochem. 2013;24:1741-1750.

D. Araújo JR, Ramalho C, Correia-Branco A, Faria A, Ferraz T, Keating E, Martel F. Parallel increase in placental oxidative stress and antioxidant defenses occurs in type 1 but not gestational diabetes. Placenta. 2013;34:1095-1098.

E. Araújo JR, Gonçalves P, Martel F. Modulation of glucose uptake in a human choriocarcinoma cell line (BeWo) by dietary bioactive compounds and drugs of abuse. J Biochem. 2008;144:177-186.

F. Araújo JR, Pereira AC, Correia-Branco A, Keating E, Martel F. Oxidative stress induced by *tert*-butylhydroperoxide interferes with the placental transport of glucose: *in vitro* studies with BeWo cells 2013. Eur J Pharmacol. doi: 10.1016/j.ejphar.2013.10.023.

G. Araújo JR, Correia-Branco A, Pereira AC, Pinho MJ, Keating E and Martel F. Oxidative stress decreases uptake of neutral amino acids in a human placental cell line (BeWo cells). Reprod Toxicol. 2013;40:76-81.

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INTRODUCTION

1. Gestational Diabetes Mellitus

1.1. Definition, Prevalence and Diagnostic Criteria

Diabetes mellitus is the most prevalent metabolic disorder affecting pregnant women [Negrato and Gomes, 2013]. Gestational diabetes mellitus (GDM) constitutes the most common type [Lappas et al., 2011], corresponding approximately to 90% of diabetic pregnancies, whereas type 1 (T1D) and type 2 diabetes account for the remaining 10% [Magon and Chauhan, 2012; Vargas et al., 2010].

GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (usually towards the late-second and early-third trimester) [ADA, 2013]. Its prevalence is difficult to compare from country to country due to the different diagnostic criteria used among distinct populations [Nolan, 2011]. In this sense, to promote the endorsement of consensual diagnostic criteria for GDM and, at the same time, to clarify the thresholds of maternal glycemia associated with an increased risk of adverse maternal, fetal and neonatal outcomes [Negrato and Gomes, 2013], the International Association of Diabetes in Pregnancy Study Group (IADPSG) Consensus Panel was created in 1998 [Metzger et al., 2010]. The results from the hyperglycemia and adverse pregnancy outcomes (HAPO) study [Metzger et al., 2008] along with other similar studies [Jensen et al., 2008; Pettitt et al., 1980; Sacks et al., 1995], served as the fundamental basis for the development of new diagnostic criteria by the IADPSG Consensus Panel. The HAPO study was a large-scale, multicentric and multiethnic epidemiologic research study which demonstrated for the first time that the risk of adverse perinatal outcomes, such as birth weight above the 90th percentile (large-for-gestational age [LGA] newborns), neonatal hyperinsulinemia, hyperbilirubinemia, hypoglycemia and adiposity, preterm delivery, cesarean section and preeclampsia, linearly increased as a function of maternal plasma glucose levels measured at 24-28 weeks of gestation. This association was present, even for glycemic ranges lower than that of overt diabetes [Metzger et al., 2008].

In 2010, the IADPSG Consensus Panel recommended that all women not previously diagnosed with diabetes before pregnancy should undergo a 75-g oral glucose tolerance test (OGTT) at 24-28 weeks of gestation. Attaining or exceeding any of the following plasma glucose thresholds would make a GDM diagnosis: 92

mg/dl (5.1 mM) for fasting plasma glucose, 180 mg/dl (10.0 mM) for 1-h OGTT plasma glucose or 153 mg/dl (8.5 mM) for 2-h OGTT plasma glucose [Metzger et al., 2010]. These new diagnostic criteria, which were adopted by Portugal in 2011 [Vicente and Boavida, 2011], significantly increased the prevalence of GDM to approximately 18% of all pregnancies (range 9.3-25.5%) [Sacks et al., 2012], which is more than the double of the prevalence based on older criteria [Homko, 2011], such as those of Carpenter and Coustan [Berggren et al., 2011]. This rise is explained by the fact that only one abnormal blood glucose value [Metzger et al., 2010], in contrast to the older criteria that stipulated at least two (reviewed in [Negrato and Gomes, 2013]), is sufficient to make a GDM diagnosis [ADA, 2013].

1.2. Etiology and Pathogenesis

From the mid-second trimester until term, pregnancy is associated with a progressive increase in insulin resistance [Ben-Haroush et al., 2004] to ensure an appropriate glucose delivery to the growing fetus [Wong et al., 2002]. This appears to result from a combination of increased maternal adiposity and circulating levels of insulin-desensitizing hormones [Ben-Haroush et al., 2004; Buchanan and Xiang, 2005], in particular human placental lactogen and growth hormone, progesterone, cortisol, prolactin and estrogen. Altered levels of adipose tissue-derived mediators, such as an increase in leptin and tumor necrosis factor-alpha (TNF-α) and a decrease in adiponectin have also been reported to contribute to pregnancy-induced insulin resistance [Golbidi and Laher, 2013; Vargas et al., 2010].

In normal pregnant women, this physiological insulin resistance is compensated by an increase in insulin secretion. However, in GDM women this compensatory insulin secretion is not sufficient to maintain response, and maternal hyperglycemia develops [Buchanan and Xiang, 2005]. So, hallmarks of GDM include insulin resistance, hyperglycemia [ADA, 2013; Ben-Haroush et al., 2004] and hyperinsulinemia [Lepercq et al., 1998]. Although not exclusive to GDM, hyperleptinemia [Ategbo et al., 2006; Guvener et al., 2012] and elevated levels of proinflammatory cytokines, such as TNF-α [Ategbo et al., 2006; Plomgaard et al., 2007], are also associated with this disease.

It is still poorly understood why the increase in insulin secretion is inadequate to accommodate the increased insulin resistance in GDM. Two of the best

characterized molecular mechanisms include: a) the presence of a defect in insulin post-receptor signaling pathway (phosphatidylinositol 3-kinase [PI3K]) in peripheral maternal tissues, thereby contributing to a reduced insulin-mediated glucose uptake [Friedman et al., 1999; Metzger et al., 2007], and b) a defect in pancreatic β-cell function [Metzger et al., 2007]. On the other hand, since GDM is a multifactorial disorder, several modifiable and non-modifiable factors may also explain the increased risk of developing this disease [Ben-Haroush et al., 2004]. These factors include: a) maternal overweight or obesity (pre-gravid body mass index ≥ 25) [Chu et al., 2007], b) advanced maternal age [Khalil et al., 2013], c) high parity (number of times a women has given birth) [Ben-Haroush et al., 2004], d) adverse obstetric outcomes in previous pregnancies (eg. stillbirth, LGA newborns and GDM) [Buchanan and Xiang, 2005] and e) diabetes in first-degree relative(s) [Buchanan and Xiang, 2005].

1.3. Perinatal and Long-term Outcomes

As described in HAPO study (briefly reviewed in section 1.1.), GDM is associated with perinatal complications, in particular fetal macrosomia, which increases the risk of LGA newborn deliveries and traumatic birth injuries [Metzger et al., 2010; Metzger et al., 2008]. Furthermore, GDM increases the risk of the newborn to develop cardiovascular and metabolic diseases (obesity, type 2 diabetes and metabolic syndrome) [Boney et al., 2005; Gluckman et al., 2008; Lee et al., 2007] and neurological dysfunctions (inattention, hyperactivity and lower intelligence quotient scores) later in life [Ornoy, 2005; Stenninger et al., 1998]. Altogether, this data support the importance of GDM in fetal programming of adult diseases.

Also, although most GDM women return to normal glucose tolerance after delivery [Kautzky-Willer et al., 2001], they present an increased risk of developing type 2 diabetes and having a cardiovascular event later in life [Metzger et al., 2007].

Maternal hyperglycemia is recognized as the main risk factor for the occurrence of adverse pregnancy outcomes associated with GDM [Biri et al., 2009; Pettitt et al., 2008; Simeoni and Barker, 2009; Yessoufou and Moutairou, 2011]. However, poor outcomes have also been reported in infants born from GDM mothers with an adequate glycemic control [Franks et al., 2006; Takayama-Hasumi et al., 1994], suggesting that, besides hyperglycemia, other GDM-associated

conditions, such as hyperinsulinemia, hyperleptinemia, inflammation and also oxidative stress - a condition in which cellular production of reactive oxygen species (ROS) overwhelms the ability of the antioxidant system to maintain ROS within physiological levels [Kohen and Nyska, 2002; Lappas et al., 2011] – may also be involved in GDM pathophysiology. In fact, oxidative stress could adversely affect insulin production by the pancreas leading to β-cell apoptosis, further exacerbating GDM metabolic phenotype [Herrera and Ortega-Senovilla, 2010].

The cellular and molecular mechanisms by which GDM and its conditions increase perinatal and later in life adverse health outcomes are still largely unexplored. However, some hypothesis have been raised, including changes in nutrient supply to the fetus, particularly fatty acids, amino acids and micronutrients [Metzger et al., 2007]; alterations in the expression of genes that control fetal growth and development by epigenetic mechanisms [Simeoni and Barker, 2009]; and alterations in placental development [Simeoni and Barker, 2009].

Understanding how these mechanisms are altered in GDM will certainly contribute for a better understanding of GDM pathophysiology and to unveil pharmacological and nutritional strategies to prevent the occurrence of GDM complications.

1.4. Management

Antepartum care is absolutely essential to normalize maternal glycemia and to reduce the morbidity and mortality associated with GDM [Buchanan and Xiang, 2005; Faraci et al., 2011]. Nutritional therapy is the first-line treatment [Metzger et al., 2007] but if adequate glycemic control is not achieved, subcutaneous insulin therapy is initiated. Physical exercise is also recommended for all GDM women capable of performing it [Golbidi and Laher, 2013].

Due to the similar effectiveness, but easier administration and lower cost when compared to insulin, selected oral anti-diabetic drugs (gliburyde and metformin) can be used as an alternative to insulin therapy [Relatório de Consenso sobre a Diabetes e Gravidez, 2011]. However, the risk/benefit of their use must be carefully assessed [Relatório de Consenso sobre a Diabetes e Gravidez, 2011], as their long-term safeness to the offspring health is still not completely proven [Nolan, 2011].

2. Human Placenta

2.1. Structure and Functions

In mammals, the placenta is the main interface between the maternal and fetal blood circulations [Fowden et al., 2008]. This transient organ of fetal origin performs multiple functions required for the growth and development of the fetus. Namely, a) it anchors the conceptus to the uterine wall and prevents its rejection by the maternal immune system [Fowden et al., 2008; Sandovici et al., 2012], b) it mediates the exchange of nutrients, respiratory gases, water and waste metabolites between maternal and fetal blood [Fowden et al., 2008] and c) it produces and metabolizes several hormones (including prolactin, progesterone, lactogen, growth hormone, chorionic gonadotropin and leptin), growth factors, cytokines and eicosanoids, that can be secreted into both the fetal and maternal circulations [Fowden et al., 2008; Fowden et al., 2009; Sandovici et al., 2012].

The human term placenta is constituted by three tissue layers: a) the syncytiotrophoblast (STB) epithelium, which is supported by an underlying basement membrane, b) the fetal connective tissue and c) the fetal capillary endothelium (Fig. 1) [Cleal and Lewis, 2008; Jansson et al., 2009].

During the first 21 days of pregnancy, trophoblast cells differentiate into villous or extravillous cytotrophoblasts. The extravillous cytotrophoblasts invade the myometrium and uterine vasculature, being essential for the anchorage of the placenta to the uterus, whereas villous cytotrophoblasts differentiate and fuse originating the STB layer [Malassine et al., 2003]. This layer has two polarized plasma membranes: the microvillous membrane (MVM), directed towards the maternal blood, and the basal membrane (BM), facing fetal capillaries [Jansson et al., 2009] (Fig. 1). Since maternal blood comes into direct contact with the STB the human placenta is designated as hemochorial [Carter, 2012]. This placental type is also typical of many rodents and primates.

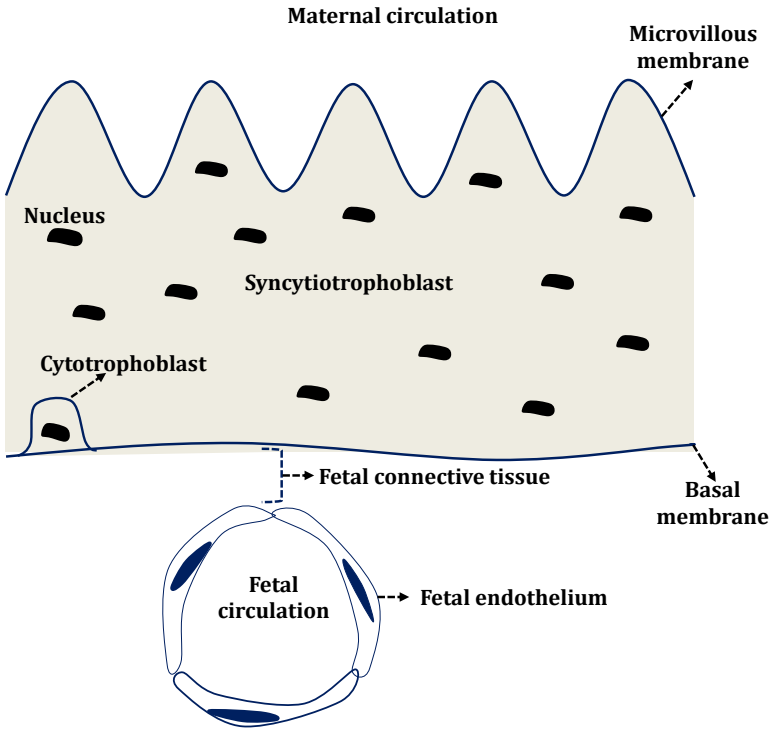

Fig. 1. Representation of the human term placenta structure at the maternal-to fetal interface.

The STB epithelium is the most important placental tissue actively involved in the maternal-to-fetal transport of nutrients such as glucose, amino acids, fatty acids, vitamins and ions [Desforges and Sibley, 2010; Jansson et al., 2009; Sandovici et al., 2012]. Nutrient transport across the STB can occur by simple diffusion, protein-mediated transport or by receptor-mediated endocytosis [Desforges and Sibley, 2010]. The rate at which these processes occur depend mainly on the physico-chemical characteristics of the nutrient (eg. electrical charge and hydrophilicity/hydrophobicity) [Desforges and Sibley, 2010], but are also dependent on placental size, surface area, thickness and metabolism (ie. nutrient consumption and production) [Fowden et al., 2008; Lager and Powell, 2012], nutrient concentration gradient between maternal and fetal circulations [Fowden et al., 2009], and on uteroplacental and fetal vasculature and blood flow. Moreover, placental transport also depends upon the location, expression and activity of transporter proteins present in the STB plasma membranes [Fowden et al., 2008; Lager and Powell, 2012].

In vivo, transport across the human placenta can be studied by measuring the appearance of nutrients labeled with stable isotopes (eg. ^{13}C) in cord blood, after their administration to the mother [Haggarty, 2010]. However, since these studies

give us an indirect measure of transport (as it is inferred from isotope concentration differences between the maternal and fetal blood circulations), *in vitro* placental models have been developed in order to directly access transport rates [Haggarty, 2010], namely placental explants, STB membrane vesicles and cell cultures of human trophoblasts (primary cells and cell lines) [Levkovitz et al., 2013].

2.2. Placental Transport of Nutrients

The transplacental transport of nutrients is of particular importance to assure an adequate fetal growth and development [Avagliano et al., 2012]. Moreover, changes in nutrient transport capacity across the STB membranes have been associated with abnormal size and weight at birth, which, as first noted by [Barker et al., 1993], are both major determinants of perinatal morbidity and mortality and of later in life development of cardiovascular and metabolic diseases [Jansson et al., 2009; Lager and Powell, 2012].

The major subject of this study was to investigate the impact of GDM upon placental transport of the nutrients folic acid (FA), L-methionine (L-Met), arachidonic (AA) and docohexaenoic (DHA) acids and glucose. So, in the next section we will briefly describe the main principles of FA, amino acids, long-chain polyunsaturated fatty acids (LC-PUFAs) and glucose placental transport.

2.2.1. FA Transporters

FA or pteroylmonoglutamate is the parent structure and oxidized form of the B_9 family of water-soluble vitamins known as folates [Lucock, 2000]. N^5-methyltetrahydrofolate, a reduced folate, is the most common form naturally occuring in food [Lucock, 2000], and nutritional supplements and fortified foods are the most abundant dietary sources of FA [Caballero et al., 2005; Lucock, 2000].

Folates are essential micronutrients that facilitate the intracellular transfer of one-carbon units required for a) the synthesis of purine and pyrimidine precursors of nucleic acids [Lucock, 2000], b) the occurrence of methylation reactions [Farkas et al., 2013], c) the initiation of protein synthesis in the mitochondria and d) the metabolism of some amino acids (L-Met, L-Serine [L-Ser], Glycine and L-Histidine) [Lucock, 2000] (Fig. 2). Due to this, folates play a crucial role in the process of cell division and proliferation, being thus particularly

important during pregnancy to assure placental and fetal development and uterine expansion [Caballero et al., 2005; Lucock, 2000].

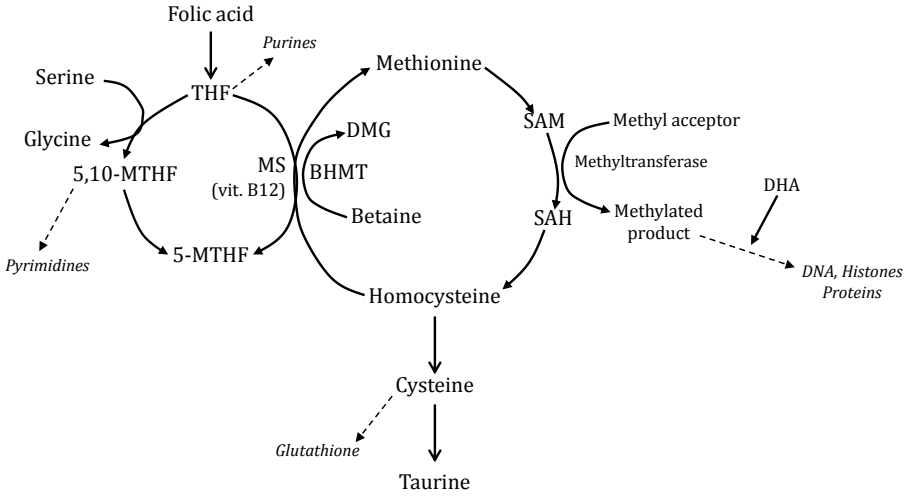

Fig. 2. Interaction of folic acid, methionine and docosahexaenoic acid in one carbon cycle. 5,10-MTHF: N^5,N^{10}-methylenetetrahydrofolate; 5-MTHF: N^5-methyltetrahydrofolate; BHMT: betaine–homocysteine methyltransferase; DHA: docosahexaenoic acid; DMG: dimethylglycine; MS: methionine synthase; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; THF: tetrahydrofolate; vit B_{12}: vitamin B_{12}.

The increased demand for folates during pregnancy is well demonstrated by the fact that maternal folate deficiency, the most common vitamin deficiency in developed countries [Caballero et al., 2005], is associated with an increased risk of low birth weight, spontaneous abortion, and neural tube defects (NTDs) (eg. spina bifida and anencephaly) [Caballero et al., 2005; Lucock, 2000; Zhao et al., 2011]. Moreover, FA supplementation during the periconceptional period reduces the incidence of such outcomes, particularly of NTDs [Caballero et al., 2005; CDC, 1991; Lucock, 2000].

Neither the placenta nor the fetus is able to synthesize folates, so this vitamin must be obtained from the maternal circulation through placental transport [Giugliani et al., 1985; Hutson et al., 2012]. The human placenta expresses three specific folate transporters, namely the reduced folate carrier (RFC1), the alpha isoform of the folate receptor (FRα) and the proton-coupled folate transporter (PCFT). All of them act coordinately to ensure the vectorial transfer of folates from

maternal to fetal circulation [Keating et al., 2009a; Solanky et al., 2010; Zhao et al., 2009] (Fig. 3).

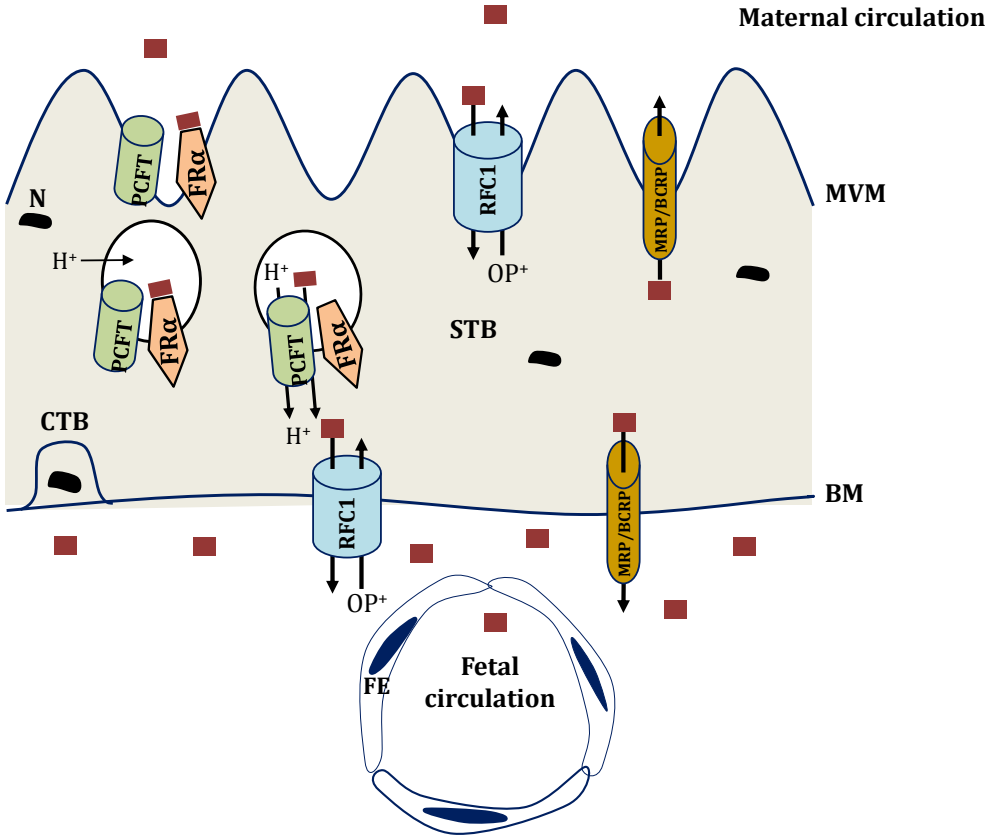

Fig. 3. Main folic acid transporters present at the human syncytiotrophoblast.

■: folic acid; BM: basal membrane; CTB: cytotrophoblast; FRα: folate receptor alpha; FE: fetal endothelium; MVM: microvillous membrane; N: nucleus; OP^+: organic phosphates; PCFT: proton-coupled folate transporter; STB: syncytiotrophoblast; RFC1: reduced folate carrier.

RFC1 is a folate:organic phosphate exchanger that utilizes the transmembrane organic phosphate gradient to mediate the uphill transport of folates into cells [Zhao et al., 2011; Zhao et al., 2009]. This bidirectional transporter is expressed in both MVM and BM of the STB [Solanky et al., 2010] (Fig. 3), has a maximal activity at physiological pH, and a higher affinity for reduced folates (such as N^5-methyltetrahydrofolate) over non-reduced folates [Zhao et al., 2011; Zhao et al., 2009]. FRα is a high-affinity folate-binding protein selectively expressed in the MVM of the STB (Fig. 3). This transporter is embedded in the membrane by a glycosylphosphoinositol anchor [Solanky et al., 2010; Zhao et al., 2011; Zhao et al., 2009] and mediates the unidirectional uptake of folates at neutral to mildly acidic

pH. FRα has a higher affinity for non-reduced over reduced folates [Zhao et al., 2011; Zhao et al., 2009]. Concerning PCFT, this transporter is a high affinity folate:H^+ symporter, with an optimal activity at acidic pH (5.5–6.0), that utilizes the transmembrane H^+ gradient to achieve the uphill transport of folates into cells [Keating et al., 2009a; Zhao et al., 2011; Zhao et al., 2009]. PCFT is predominantly present at the MVM, where it co-localizes with FRα [Solanky et al., 2010] (Fig. 3).

Recently, Solanky and co-workers proposed a model for folates transport across the human STB (Fig. 3). The initial step involves binding of folates to FRα localized in the MVM. Co-localization of this transporter with PCFT allows the internalization of both into an endosome. During cytoplasmic transit, this endosome is acidified (pH 6.0–6.5) due to an influx of protons, which promotes the dissociation of folates from FRα and establishes a favourable H^+ gradient that allows PCFT-mediated folate efflux into the cytoplasm. FRα and PCFT are then re-cycled back to the MVM surface. At the MVM, RFC1 provides an alternative pathway for folates uptake. Efflux across the BM does not involve neither FRα nor PCFT but instead RFC1.

Other potential folate transporters localized at BM and MVM of the STB (namely the ATP-binding cassette efflux transporters: multidrug resistance-associated proteins and breast cancer resistance protein [Zhao et al., 2011]), may also play a role in FA transport but their exact contribution is still poorly understood.

2.2.1.1. Regulation of placental FA transport

Despite the recognized importance of folates for fetal development, knowledge on the cellular mechanisms involved in its transfer across the placenta is still very limited. Available data indicates that placental uptake of FA is downregulated by chronic hyperglycemia, some anti-hypertensive drugs, drugs of abuse and ethanol [Keating et al., 2009a], and is differently modulated by polyphenolic compounds and methylxanthines [Keating et al., 2008]. Although maternal folates deficiency and low birth weight are intimately associated, a decrease in folates placental transport has not been demonstrated in FGR [Bisseling et al., 2004]. Instead, an increase was reported by Keating and co-workers, which

suggested that the placenta exhibited “a compensation for the weakness effect" [Keating et al., 2009b].

2.2.2. Amino acid Transporters

Besides being used as building blocks for fetal protein synthesis, amino acids constitute important energy substrates for both the fetus and the placenta [Jansson et al., 2009] and they are biosynthetic precursors of purines, pyrimidines, neurotransmitters, nitric oxide, glutathione, polyamines and haem [Cleal and Lewis, 2008; Grillo et al., 2008]. By virtue of not being synthesized by the fetus or placenta, placental transport of nutritionally essential amino acids from the maternal circulation is determinant for these functions to occur.

L-Met is a nutritionally essential large neutral amino acid that plays a key role in one-carbon metabolism. After conversion to S-adenosylmethionine, this amino acid provides the methyl groups necessary for the methylation of DNA, RNA, proteins, biogenic amines and phospholipids (Fig. 2). The importance of L-Met during pregnancy is well demonstrated by the higher occurrence of NTDs in women with low dietary intake of L-Met, and FGR in animal models of L-Met intake restriction during pregnancy [Kalhan and Marczewski, 2012].

Epigenetic regulation, in particular DNA methylation, plays a crucial role in gene expression, imprinting processes and embryonic development, thereby programming the fetus for future development of diseases [Jansson and Powell, 2007]. Since biological methylation reactions depend on the availability of L-Met [Mato et al., 2008], and also of FA, changes in the placental transport of these methyl-related nutrients will alter their availability to the fetus, providing a direct link between placental function, gene methylation and fetal programming [Jansson and Powell, 2007].

The concentration of most amino acids, including L-Met, at the placental level is normally higher than that found in both fetal and maternal circulation [Cleal and Lewis, 2008]. This suggests that their uptake across the MVM into the STB occurs mainly by active processes [Desforges and Sibley, 2010], whereas their transport across the BM into the fetal blood is mainly passive [Burton et al., 2011].

Based on the functional characteristics of amino acid transport systems, such as substrate specificity, inhibition by L-Met and placental location, four different

transporters have been identified in the human STB as capable of transporting L-Met: systems A, L, y^+L and b^{0+} [Cleal and Lewis, 2008; Kudo and Boyd, 1990; Tsitsiou et al., 2009] (Fig. 4).

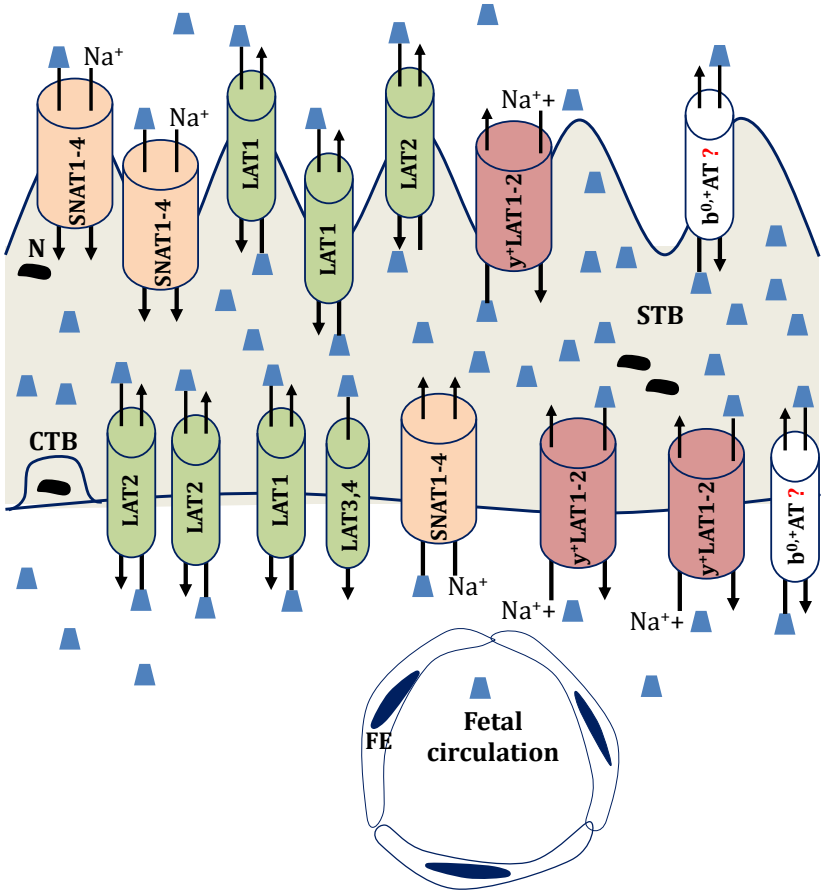

Fig. 4. Main L-methionine transport systems present at the human syncytiotrophoblast.
: amino acids; BM: basal membrane; CTB: cytotrophoblast; FE: fetal endothelium; LAT: L-type amino acid transporter; MVM: microvillous membrane; N: nucleus; SNAT: sodium coupled neutral amino acids transporter; STB: syncytiotrophoblast; ?: expression not confirmed.

The Na^+-dependent system A transporters mediate the uptake of neutral amino acids (both essential and non essential) with short and unbranched side chains, mostly L-Alanine (L-Ala), Glycine, L-Ser, L-Met and L-Glutamine [Cleal and Lewis, 2008; Desforges and Sibley, 2010]. Although system A activity is present in both MVM and BM of the STB, its activity is highly polarized to the MVM [Jansson et al., 2001]. In agreement with this, the system A isoforms sodium-coupled neutral amino acids transporter 1 (SNAT1), SNAT2 and SNAT4 are expressed mainly in the MVM in comparison with the BM [Burton et al., 2011] (Fig. 4). SNAT1-4 are accumulative transporters that mediate the influx of amino acids from maternal

circulation into the STB, creating an outwardly directed concentration gradient that will enable them to be transported outside the STB, towards the fetal circulation, by using other amino acid transport systems.

System L is a Na^+-independent amino acid transport system and the main route for the transport of many neutral (eg. L-Met), branched-chain (eg. L-Leucine) and aromatic (eg. L-Phenylalanine and L-Tyrosine) amino acids [Burton et al., 2011; Cleal and Lewis, 2008]. Cellular localization of system L isoforms is polarized, with L-type amino acid transporter 1 (LAT1) and LAT2 being primarily, although not exclusively, expressed in the MVM and BM, respectively (Fig. 4). Both of them allow the exchange of intracellular non-essential amino acids (eg. transported by system A) for extracellular essential amino acids, driving the uptake into STB of essential amino acids [Burton et al., 2011]. Additionally, LAT3 and LAT4 system L isoforms, which are expressed in the BM of the STB, act as efflux transporters of amino acids from the STB to the fetal circulation [Lager and Powell, 2012] (Fig. 4).

Besides the neutral amino acids transport systems mentioned above (systems A and L), placental transport of L-Met also appears to involve the cationic amino acid transport systems y^+L and $b^{o,+}$.

System y^+L (represented by y^+LAT1 and y^+LAT2 amino acid transporters) have been described to be present in both MVM and BM of the human STB (Fig. 4); however, it is much more abundant at the BM. This system has been described to exchange cationic amino acids (eg. L-Lysine, L-Lys), which are transported out of the STB down their electrical potential gradient, for neutral amino acids (eg. L-Leucine, L-Met) plus Na^+, which are transported into the STB [Grillo et al., 2008]. System $b^{o,+}$ (isoform $b^{0,+}AT$) has similar characteristics as y^+L, except that it transports neutral amino acids in the absence of Na^+ [Battaglia and Regnault, 2001; Cleal and Lewis, 2008; Jansson, 2001]. More detailed information about system $b^{o,+}$ activity and expression in the STB membranes is at the moment scarce and conflicting [Ayuk et al., 2000; Jansson, 2001] (Fig. 4).

2.2.2.1. Regulation of placental amino acids transport

In placentas obtained from FGR newborns, the activity and expression of systems A, L and y^+L, and of cationic amino acid transporter system y^+ and aromatic amino acid transporter system T were shown to be reduced in MVM and/or BM of

the STB (reviewed by [Avagliano et al., 2012; Larque et al., 2013]). On the other hand, in fetal overgrowth [Jansson et al., 2013; Jansson et al., 2002] the activity and expression of system A transporters appears to be increased in MVM. Collectively, these data show that reduced fetal growth is associated with a downregulation whereas fetal overgrowth appears to be associated with an upregulation of placental amino acid transporters. Whether these alterations are a cause or a consequence of impaired fetal growth, is at the moment difficult to address [Cleal and Lewis, 2008].

The placental transport of amino acids is regulated by several maternal and fetal factors [Cleal and Lewis, 2008]. Hormones (insulin and leptin) growth factors (insulin-like growth factors) [Cleal and Lewis, 2008; Fowden et al., 2009], proinflammatory cytokines (interleukin (IL)-6 and TNF-α) and glucocorticoids (cortisol) [Burton et al., 2011; Fowden et al., 2009] have been shown to increase system A transporter activity and SNAT1 and 2 expression. Conversely, adiponectin, IL-1β, angiotensin II, hypoxia and oxidative stress reduce system A activity and/or SNAT2 expression (reviewed by [Burton et al., 2011; Lager and Powell, 2012]).

System L is only affected by adiponectin, which downregulates its activity [Rosario et al., 2012] and by insulin, which either increases or does not affect its activity (Lager and Powell, 2012). The intracellular signaling pathway mammalian target of rapamycin (mTOR) acts as a positive regulator of both systems A and L [Jansson et al., 2012; Rosario et al., 2013].

2.2.3. LC-PUFAs Transporters

The nutritionally essential LC-PUFAs AA (20:4n-6) and DHA (22:6n-3) play a fundamental role for both the health of the pregnant woman and for the growth and development of the fetus [Innis, 1991]. Apart from serving as an energy substrate, LC-PUFAs are essential components of cellular membranes, maintaining their structure and function, and act as regulators of gene expression via nuclear receptors [Schmitz and Ecker, 2008] and DNA methylation [Kulkarni et al., 2011] (Fig. 2).

AA is essential for the synthesis of eicosanoids such as prostaglandins and leukotrienes, which are important for the development of fetal nervous, visual, immune and vascular systems [Cunningham and McDermott, 2009; Duttaroy, 2009] and for labour [Allen and Harris, 2001]. DHA is also essential for the development of

the fetal neurovisual system, being incorporated in high concentrations in the brain and retina [Cunningham and McDermott, 2009; Duttaroy, 2009]. Through the conversion of phosphatidylethanolamine-DHA to phosphatidylcholine-DHA, DHA levels were shown to regulate methyl group availability [Kulkarni et al., 2011] (Fig. 2). A depletion of both AA and DHA in fetal tissues is associated with cognitive, behavioral and visual abnormalities later in life [Innis, 2005]. Accordingly, several studies suggest that maternal LC-PUFAs supplementation during pregnancy improves neurodevelopmental functions of the infants (reviewed in [Larque et al., 2011; Ryan et al., 2010]) and, at the same time, reduces the risk of preterm delivery [Greenberg et al., 2008] and low birth weight [Carlson et al., 2013; Greenberg et al., 2008; Larque et al., 2012]. Based on this, the European Food Safety Authority (EFSA) recommends that maternal intake of LC-PUFAs should be increased during pregnancy and lactation [EFSA, 2010].

Optimal fetal growth and development depends on an adequate supply of AA and DHA from maternal circulation [Duttaroy, 2009; Haggarty, 2010; Johnsen et al., 2009]. Interestingly, most LC-PUFAs are present at higher concentrations in fetal than in maternal circulation [Hanebutt et al., 2008] - a phenomenon termed biomagnification [Larque et al., 2011]. This is thought to be the result of a selective and more efficient placental transport of LC-PUFAs, over non-essential shorter fatty acids, in favor of the fetus [Haggarty, 2010].

LC-PUFAs transported across the human STB are mainly derived from triglyceride-rich lipoproteins (from which they must be released by the action of placental lipases) and from fatty acids bound to albumin [Hanebutt et al., 2008] (Fig. 5). Although LC-PUFAs may be taken up by the STB via passive diffusion, membrane-associated fatty acid transport proteins are also implicated in this transport process. Namely, placental plasma membrane fatty acid binding protein (pFABPpm), fatty acid transport proteins (FATP) and fatty acid translocase (FAT/CD36) [Duttaroy, 2009; Haggarty, 2010]. Additionally, intracellular fatty acid binding proteins (FABP), in particular FABP 1, 3 and 4, are responsible for directing fatty acids to their specific intracellular locations [Cunningham and McDermott, 2009; Duttaroy, 2009] (Fig. 5). The precise mechanisms by which these transport proteins facilitate transmembrane passage of LC-PUFAs are still a matter of speculation.

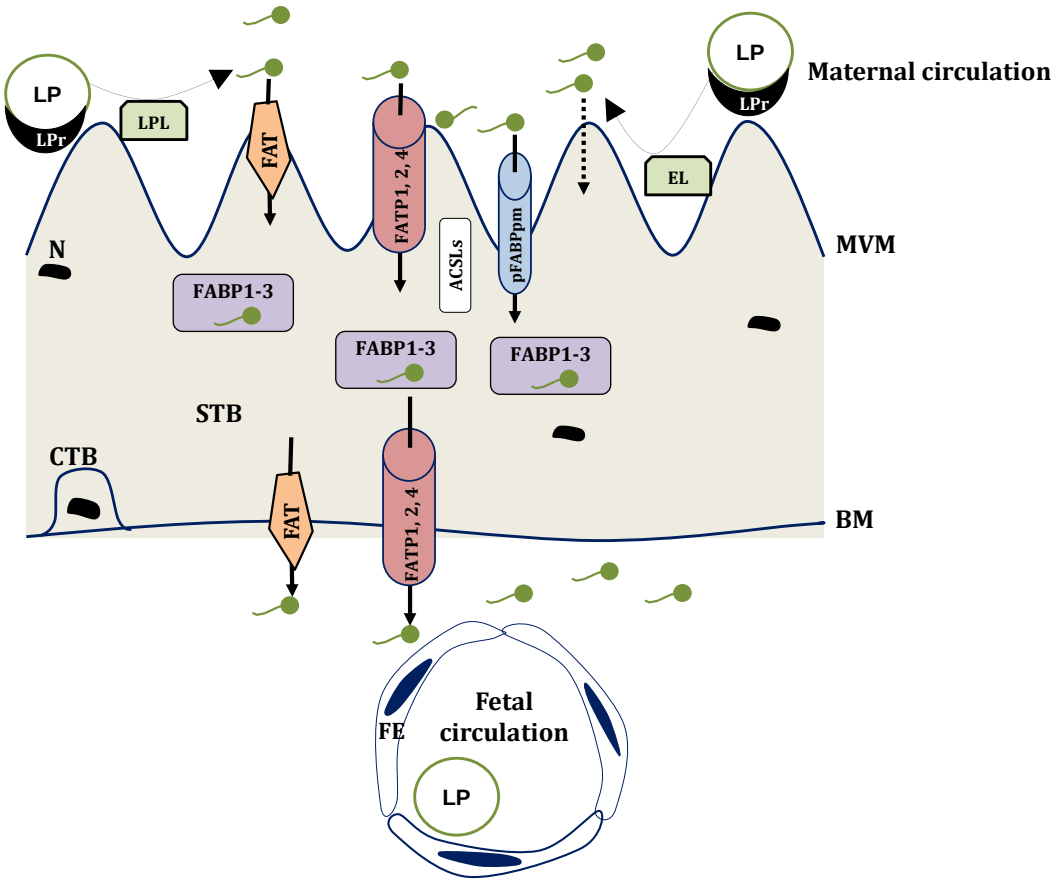

Fig. 5. Long-chain polyunsaturated fatty acids transport at the human syncytiotrophoblast.
: long-chain polyunsaturated fatty acid; ACSL: long-chain acyl-CoA synthetases; BM: basal membrane; CTB: cytotrophoblast; FATP: fatty acid transport proteins; FAT/CD36: fatty acid translocase; EL: endothelial lipase; FE: fetal endothelium; LP: lipoprotein; LPL: lipoprotein lipase; LPr: lipoprotein receptor; MVM: microvillous membrane; N: nucleus; pFABPpm: placental plasma membrane fatty acid binding protein; STB: syncytiotrophoblast.

pFABPpm is an unidirectional placenta-specific transporter with preferential affinity for DHA and AA, that is found exclusively on the MVM of term STB (Fig. 5) [Cunningham and McDermott, 2009; Duttaroy, 2009].

The FATP family (FATP1–6) transports fatty acids in an ATP-dependent manner with no preference for any particular LC-PUFA [Duttaroy, 2009; Richards et al., 2003], and FATP1, 2 and 4 are expressed in the MVM and BM of the human STB [Cunningham and McDermott, 2009] (Fig. 5). A still ongoing debate about FATP is whether they act solely as transmembrane transport proteins or if they also harbor long-chain acyl-CoA synthetase (ACSL) activity [Bonen et al., 2007; Duttaroy, 2009]. ACSL are a group of cytosolic enzymes that trap and prevent the efflux of

intracellular fatty acids by converting them into acyl-CoA derivatives for further esterification or β-oxidation [Pohl et al., 2004; Weedon-Fekjaer et al., 2010]. These enzymes, in particular ACSL1, 3, 4, 5 and 6, have been proven to be involved in LC-PUFAs uptake by trophoblasts [Duttaroy, 2009; Haggarty, 2010; Johnsen et al., 2009].

FAT/CD36 is a glycoprotein receptor that allows bididirectional and non-selective transport of fatty acids, being expressed in both membranes of the STB (Fig. 5) [Haggarty, 2010]. At the present, it still arguable if FAT/CD36 is really important for AA and DHA placental transport [Cunningham and McDermott, 2009].

2.2.3.1. Regulation of placental transport of LC-PUFAs

Alterations in placental LC-PUFAs transport may occur in pregnancies complicated by FGR, obesity or diabetes. Fetal:maternal blood ratio of AA and DHA was found to be lower in FGR pregnancies compared with normal pregnancies [Hanebutt et al., 2008]. Despite FAT/CD36 [Laivuori et al., 2006] and FABP1 and 3 [Cunningham and McDermott, 2009] gene expression being unchanged in placentas from growth restricted fetuses, the activity of lipoprotein lipase was found to be decreased, thereby reducing the availability of free fatty acids to be transported by the STB [Magnusson et al., 2004b].

Recently, Dube et al. suggested that maternal obesity differently modulates placental LC-PUFAs uptake: a decrease in FATP4 expression and trophoblast linoleic acid transport but, at the same time, an increase in CD36 expression [Dube et al., 2012].

An upregulation in the expression levels of FABP1, 3 and 4 [Magnusson et al., 2004b; Radaelli et al., 2009] and ACSL2, 3 and 4 [Radaelli et al., 2009] have been described in GDM and T1D placentas, suggesting an increase in LC-PUFAs placental accumulation [Gauster et al., 2011; Magnusson et al., 2004b]. On the other hand, plasma levels of AA and DHA in neonates born from T1D, type 2 diabetes and GDM women (reviewed by [Herrera and Ortega-Senovilla, 2010]) was lower than those born from control woman. This apparently inconsistent data suggests that placental LC-PUFAs transport in diabetic pregnancies may be impaired [Herrera and Ortega-Senovilla, 2010]. So, transport capacity at the STB level needs to be assessed in diabetic pregnancies to better understand this point.

2.2.4. Glucose Transporters

Glucose is the principal energy substrate for the feto-placental unit and, together with amino acids, constitutes the primary stimuli for fetal secretion of the growth-promoting hormone insulin [Jansson et al., 2009].

Placental transport of glucose occurs mainly through facilitative glucose transporters (GLUT) [Day et al., 2013]. At least four different GLUT isoforms are expressed in the human STB: GLUT1, 3, 4 and 12. However, the primary isoform responsible for glucose transport across both membranes of the STB is believed to be GLUT1 [Baumann et al., 2002; Carter, 2012; Jansson et al., 2009] (Fig. 6). GLUT1 distribution in the STB is asymmetric, with a greater expression and activity in the MVM, which assures that glucose is transported down its concentration gradient from maternal to fetal circulation [Baumann et al., 2002; Carter, 2012]. This led to the proposal that GLUT-mediated transport across the BM of the STB constitutes the rate limiting step in placental glucose transfer [Lager and Powell, 2012] (Fig. 6).

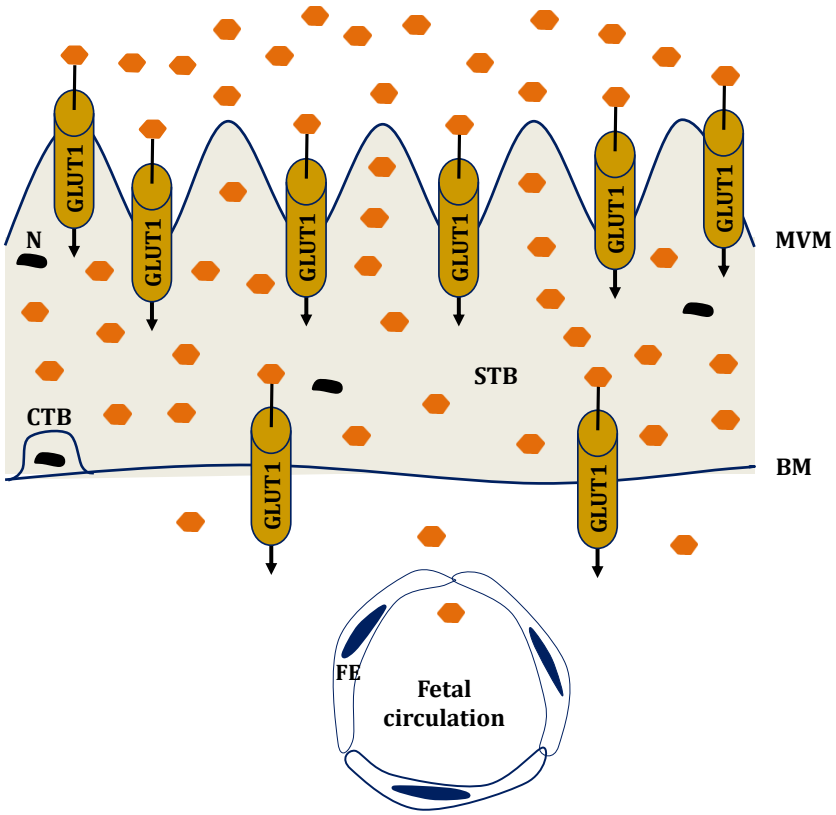

Fig. 6. Glucose transport at the human syncytiotrophoblast.

: glucose; BM: basal membrane; CTB: cytotrophoblast; FE: fetal endothelium; GLUT1: facilitative glucose transporter isoform 1; MVM: microvillous membrane; N: nucleus; STB: syncytiotrophoblast.

Glucose transporters other than GLUT1, namely GLUT3 and the insulin-sensitive GLUT4 and 12, are found in first trimester STB, and play an important role earlier in gestation [Baumann et al., 2002; Brown et al., 2011; Lager and Powell, 2012]. In accordance with this, insulin was shown to stimulate glucose transport in first trimester but not in term placentas [Challier et al., 1986; Ericsson et al., 2005]. However, this point is still not completely clarified [Baumann et al., 2002].

2.2.4.1. Regulation of placental glucose transport

Alterations in placental glucose transport and metabolism have been reported to occur in pregnancy disorders associated with aberrant fetal growth [Baumann et al., 2002; Desoye et al., 2011; Illsley, 2000; Magnusson et al., 2004a].

In T1D and GDM women, GLUT1 expression and activity was shown to be increased in the BM of the STB [Baumann et al., 2002; Gaither et al., 1999] whereas in growth restricted fetuses, GLUT1 expression and activity were unaltered [Baumann et al., 2002] but placental glucose metabolism was decreased [Magnusson et al., 2004a].

Some metabolic conditions have also been reported to regulate placental glucose transport in human trophoblasts [Baumann et al., 2002]. An inverse relationship was demonstrated between extracellular glucose concentration and GLUT1 expression and activity [Hahn et al., 1998; Illsley et al., 1998], suggesting a downregulation in GLUT1 in response to excess glucose. Additionally, corticotrophin-releasing hormone [Gao et al., 2012], insulin-like growth factor-1, growth hormone and hypoxia (reviewed by [Baumann et al., 2002]) upregulated, whereas glucocorticoids downregulated [Fowden et al., 2009] GLUT1 expression.

2.3 The Placenta as a nutrient sensor

The placenta has been proposed to acts as a nutrient sensor, by regulating the transport capacity of nutrients in response to maternal or fetal stimuli [Jansson and Powell, 2013]. According to this model, in the presence of a 'low nutritional environment', like maternal under-nutrition (which induces FGR), the fetus signals the placenta to upregulate nutrients transport (eg. folic acid, see section 2.2.1.1.) through nutrient sensing signaling pathways, such as mTOR. However, this is

inconsistent with data showing that placental transport of amino acids is downregulated in FGR (see section 2.2.2.1.) [Jansson and Powell, 2013]. As a whole, these obervations show that the nutrient sensing function of the placenta is still not completely understood being presently an attractive area of research [Jansson and Powell, 2013].

AIMS

The main aim of this study was to investigate the impact of GDM and its associated hallmarks upon placental nutrient transport and development.

To accomplish this, the specific aims of this study were:

CHAPTER I - Characterization of placental nutrient transport and placental development in normal and GDM pregnancies

- to characterize and to compare the uptake of ^{3}H-FA, ^{14}C-L-Met, ^{14}C-AA and ^{14}C-DHA by primary cultured human cytotrophoblasts obtained from normal (NTB cells) and GDM pregnancies (DTB cells)

- to compare the viability, proliferation, differentiation and apoptosis of NTB and DTB cells

CHAPTER II – Assessment of a GDM-associated hallmark (oxidative stress) at placental level

- to assess oxidative stress status in GDM placentas

CHAPTER III – Modulation of placental nutrient transport by GDM-associated hallmarks

- to study the effect of oxidative stress upon the uptake of ^{3}H-deoxyglucose (^{3}H-DG) by the BeWo choriocarcinoma cell line

- to investigate the effect of hyperglycemia, hyperinsulinemia, hyperleptinemia, and increased levels of inflammation and oxidative stress upon the transport of FA, neutral amino acids and LC-PUFAs in BeWo or NTB cells

Characterization of placental nutrient transport and placental development in normal and GDM pregnancies

The information contained in this chapter is included in the following original publications:

A. Araújo JR, Correia-Branco A, Moreira L, Ramalho C, Martel F, Keating E. Folic acid uptake by the human syncytiotrophoblast is affected by gestational diabetes, hyperleptinemia, and TNF-α.

Pediatr Res. 2013;73:388-394
DOI: 10.1038/pr.2013.14.
IF: 2.67

B. Araújo JR, Correia-Branco A, Ramalho C, Gonçalves P, Pinho MJ, Keating E, Martel F. L-methionine placental uptake: characterization and modulation in gestational diabetes mellitus.

Reprod Sci. 2013;20:1492-1507
DOI: 10.1177/1933719113488442
IF: 2.06

C. Araújo JR, Correia-Branco A, Ramalho C, Keating E, Martel F. Gestational diabetes mellitus decreases placental uptake of long-chain polyunsaturated fatty acids: involvement of long-chain acyl-CoA synthetase.

J Nutr Biochem. 2013;24:1741-1750
DOI: 10.1016/j.jnutbio.2013.03.003.
IF: 4.55

Articles | *Basic Science Investigation*

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Folic acid uptake by the human syncytiotrophoblast is affected by gestational diabetes, hyperleptinemia, and TNF-α

João R. Araújo[1], Ana Correia-Branco[1], Liliana Moreira[1], Carla Ramalho[2], Fátima Martel[1] and Elisa Keating[1]

BACKGROUND: The mechanisms whereby gestational diabetes mellitus (GDM) increases the risk of fetal overgrowth and development of metabolic diseases later in life are likely to involve changes in nutrient supply to the fetus. Hence, in this work, we hypothesize that GDM may affect folic acid (FA) supply to the placenta and fetus.

METHODS: We compared ^{3}H-FA uptake by human cytotrophoblasts isolated from normal pregnancies (normal trophoblasts; NTB cells) and GDM pregnancies (diabetic trophoblasts; DTB cells) and investigated the effect of GDM hallmarks on ^{3}H-FA uptake by BeWo cells.

RESULTS: ^{3}H-FA uptake by NTB and DTB cells was time dependent and acidic pH stimulated. When compared with NTB, ^{3}H-FA uptake by DTB cells was more sensitive to acidic pH changes and to 5-methyltetrahydrofolate and pemetrexed (PTX) inhibition, indicating a proportionally greater involvement of the proton-coupled folate transporter (PCFT). A 4-h exposure of BeWo cells to lipopolysaccharide (LPS, 1–10 μg/ml) or to high levels of tumor necrosis factor-α (TNF-α, 300 ng/l) significantly reduced ^{3}H-FA uptake. Moreover, hyperleptinemic conditions (100 ng/ml leptin) decreased ^{3}H-FA uptake by BeWo cells in a time-dependent manner when compared with normoleptinemic conditions (1 ng/ml leptin).

CONCLUSION: GDM modulates ^{3}H-FA uptake by the syncytiotrophoblast, and leptin as well as TNF-α downregulate it.

The nutritionally essential folic acid (FA; vitamin B_9) is the parent structure and oxidized form of the folate family of compounds. These compounds facilitate the intracellular transfer of one-carbon units, being involved in the synthesis of purine and pyrimidine precursors of nucleic acids, the metabolism of several amino acids, and the initiation of protein synthesis in the mitochondria (1). Maternal-to-fetal transport of folates at the level of the syncytiotrophoblast epithelium is crucial for placental and fetal development and growth because neither the placenta nor the fetus can synthesize this vitamin. In fact, maternal folate deficiency has been associated with low birth weight and neural tube defects (2), and supplementation with FA during the periconceptional period reduces the incidence of such outcomes (2,3).

The human placenta expresses the reduced folate carrier (RFC1) (4), the folate receptor isoforms α (5) and β (6), and the proton-coupled folate transporter (PCFT) (7). These transporters are believed to act coordinately to ensure the vectorial transfer of folate from maternal-to-fetal circulation.

Gestational diabetes mellitus (GDM), defined as a degree of glucose intolerance with onset or first recognition during pregnancy, affects about 7% of all pregnancies (8). This condition is associated with fetal macrosomia, which increases the risk of perinatal complications, and with cardiovascular and metabolic diseases later in life both in the mother (9) and in the offspring (10). The mechanisms whereby GDM increases the risk of fetal overgrowth and development of metabolic diseases later in life are still unclear, but are likely to involve changes in nutrient supply to the fetus (11). Methyl-nutrients such as folates, vitamin B_{12}, and methionine enable cellular methylation reactions and thus epigenetic regulation of gene expression. As such, we herein hypothesize that GDM may specifically affect folate supply to the placenta and the fetus.

To test our hypothesis, we characterized FA uptake by primary cultured cytotrophoblasts isolated from human placentas of GDM-affected pregnancies (DTB cells) and compared it with FA uptake by cytotrophoblasts isolated from human placentas of uncomplicated pregnancies (NTB cells).

Hyperglycemia, hyperinsulinemia, and insulin resistance are the hallmarks of GDM. Hyperleptinemia (12) and increased inflammation (13) are also associated with this disease. Therefore, we also investigated the modulation of FA placental uptake by these GDM-associated conditions.

We verified that ^{3}H-FA uptake by DTB cells was more pH dependent at acidic pH compared with NTB cells, indicating a higher PCFT:RFC1 ratio activity in these cells. We also observed that hyperleptinemia and tumor necrosis factor-α (TNF-α) reduced FA uptake by BeWo cells.

RESULTS

Clinical, Anthropometric, and Demographic Characteristics of the Study Groups

As shown in Table 1 (14), control and GDM groups were closely matched in terms of clinical, anthropometric, and demographic data. A trend toward increased birth weight and length and placental weight was observed in the GDM group, but these differences did not reach statistical significance.

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Received 5 March 2012; accepted 25 November 2012; advance online publication 27 February 2013. doi:10.1038/pr.2013.14

Table 1. Clinical, anthropometric, and demographic data of the study groups

	Controls	GDM
Mothers		
n	11	7
Maternal age (y)	32.4 ± 1.7	33.4 ± 1.5
BMI before delivery (kg/m^2)[a]	31.2 ± 2.1	32.3 ± 1.1
Gravida (*n*)	2.5 ± 0.5	2.1 ± 0.3
Parity (*n*)	1.1 ± 0.3	1.0 ± 0.2
Mode of delivery		
Vaginal (*n* (%))	4 (36)	3 (43)
Cesarean (*n* (%))	7 (64)	4 (57)
Therapeutics of GDM (*n* (%))	–	Nutritional: 4 (57); insulin: 3 (42)
Fasting blood glucose (mmol/l)[b]	4.3 ± 0.1	4.5 ± 0.2
HbA_{1C} (%)[c]	–	5.3 ± 0.1
Periconceptional FA use (*n* (%))[d]	10 (91)[e]	4 (57)[f]
Smokers (*n* (%))	0 (0)[g]	0 (0)
Infants		
Gestational age at birth (wk)[h]	39.2 ± 0.3	39.3 ± 0.3
Birth weight (g)[i]	3,198.6 ± 122.6	3,362.9 ± 176.3
Birth length (cm)[j]	47.9 ± 0.4	49.4 ± 0.7
Placental weight (g)	590.6 ± 25.8	648.4 ± 37.6
SGA newborn (*n* (%))	SGA 1(9)	SGA 0 (0)
AGA newborn (*n* (%))	AGA 9 (82)	AGA 6 (86)
LGA newborn (*n* (%))	LGA 1 (9)	LGA 1 (14)
Gender (*n* (%))		
Male	3 (27)	3 (43)
Female	8 (73)	4– (57)
5-min Apgar score	9.1 ± 0.2	9.0 ± 0.2

Values represent means ± SEM.

[a]Parameter unknown for two subjects (one from each group). [b]Values obtained at 24–28 wk of gestation. Parameter unknown for three subjects from the control group. [c]Values obtained at 35–36 wk of gestation. Parameter unknown for the subjects from control group because this assay is not typically ordered for subjects with no history of glucose mismanagement. [d]Dosage and initiation period unknown. [e]Parameter unknown for one subject. [f]Parameter unknown for three subjects. [g]Parameter unknown for one subject. [h]Gestational age: number of completed weeks at the time of delivery, determined by prenatal ultrasound at 11–13 wk. [i]Birth weight was evaluated to the nearest gram. [j]Length was evaluated to the nearest tenth of a centimeter after birth.

AGA, adequate for gestational age; FA, folic acid; GDM, gestational diabetic mellitus; LGA, large for gestational age, classified according to published references standards (14); SGA, small for gestational age.

^{3}H-FA Apical Uptake by NTB and DTB Cells Is Time and pH Dependent

To determine the time course of accumulation of ^{3}H-FA in NTB and DTB cells, cells were incubated at pH 5.5 with 20 nmol/l ^{3}H-FA for various periods of time. As shown in Figure 1, both NTB and DTB cells accumulate ^{3}H-FA in a time-dependent manner. In DTB cells, the rates of both inward and outward transport (k_{in} and k_{out}, respectively) were significantly higher than those for NTB cells, but the accumulation at steady state (A_{max}) was unaltered (Table 2).

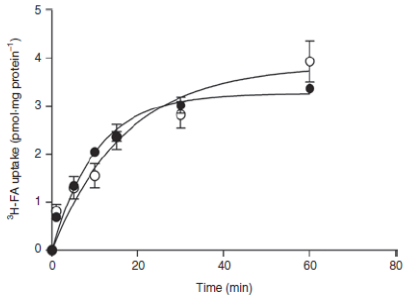

Figure 1. Time-course of ^{3}H-FA apical uptake by NTB (open circles) and DTB (solid circles) cells. Cells were incubated at pH 5.5 with 20 nmol/l ^{3}H-FA for various periods of time (n = 6–8, from three or four distinct placentas). Values shown are arithmetic means ± SEM. DTB, diabetic trophoblasts; FA, folic acid; NTB, normal trophoblasts.

Apical uptake of ^{3}H-FA by NTB cells was linear with time for up to 6 min of incubation. Therefore, a 6-min incubation time was selected for subsequent experiments.

The effect of extracellular pH on the uptake of ^{3}H-FA was examined by varying the pH of the extracellular media from 5.0 to 8.0 (Figure 2). ^{3}H-FA uptake in both NTB and DTB cells was found to be markedly acidic-pH stimulated. Strikingly, pH-dependence was greater in DTB cells, particularly for acidic pH values (5.0–6.0).

Uptake was also found to be saturable in both NTB and DTB cells but there were no differences in the evaluated kinetic parameters, maximal velocity (V_{max}), and Michaelis constant (K_m) (data not shown).

^{3}H-FA Uptake by NTB and DTB Cells Is Differentially Modulated by Folate Analogs and by SITS

To compare the specificity of the carrier process involved in ^{3}H-FA uptake in NTB and DTB cells, we determined the effect of an excess concentration of FA and its structural analogs 5-methyltetrahydrofolate, methotrexate (amethopterin), and pemetrexed (PTX) and of 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid disodium salt hydrate (SITS; an anion transport inhibitor) and thiamine pyrophosphate (TPP, a known inhibitor of RFC1) upon the uptake of ^{3}H-FA by NTB and DTB cells at pH 5.5 (Figure 3). Most of these compounds were able to similarly reduce ^{3}H-FA uptake in NTB and DTB cells. The exceptions were SITS, which slightly inhibited uptake by 12% in NTB but not in DTB cells; 5-methyltetrahydrofolate and PTX, which caused a greater inhibition of ^{3}H-FA uptake in DTB cells; and TPP, which was devoid of effect in NTB and DTB cells.

Hyperleptinemia and Inflammatory Markers Modulate ^{3}H-FA Uptake by BeWo Cells

The next set of experiments aimed to investigate the effect of specific GDM molecular hallmarks on ^{3}H-FA uptake by placental cells. For this purpose, we investigated the short- (1 and 4 h) and long-term (24 h) effects of increasing concentrations of

Table 2. Time-course parameters of ^{3}H-FA uptake by NTB and DTB cells

	n	A_{max} (pmol·mg protein^{-1})	P	k_{in} (pmol·mg protein^{-1}·min^{-1})	P	k_{out} (min^{-1})	P
NTB	8	3.84 ± 0.34		0.23 ± 0.03		0.059 ± 0.012	0.033
DTB	6	3.27 ± 0.10	NS	0.32 ± 0.02	0.039	0.097 ± 0.009	

Cells were incubated at pH 5.5 with 20 nmol/l ^{3}H-FA for various periods of time (n = 6–8, from 3 to 4 distinct placentas). Values represent means ± SEM.

A_{max}, accumulation at steady state; DTB, diabetic trophoblasts; FA, folic acid; k_{in}, constant for inward transport; k_{out}, constant for outward transport; NS, not significant; NTB, normal trophoblasts.

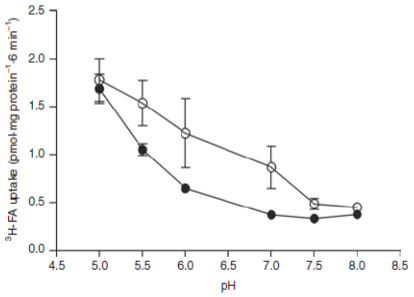

Figure 2. pH-dependence of ^{3}H-FA apical uptake by NTB (open circles) and DTB (solid circles) cells. Initial rates of uptake were determined in cells incubated with 20 nmol/l ^{3}H-FA for 6 min at extracellular pH ranging from 5.0 to 8.0. Values shown are arithmetic means ± SEM (n = 4–8, from two to three distinct placentas). Two-way ANOVA retrieved significance values for the pH (P < 0.0001) and the GDM (P = 0.0026) effects. FA, folic acid; GDM, gestational diabetes mellitus.

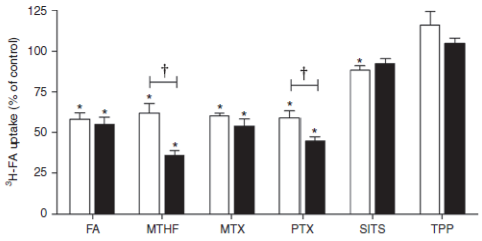

Figure 3. Effect of folic acid (FA; 100 µmol/l), 5-methyltetrahydrofolate (MTHF; 10 µmol/l), methotrexate (MTX; 10 µmol/l), pemetrexed (PTX; 20 µmol/l), 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid disodium salt hydrate (SITS; 500 µmol/l), and thiamine pyrophosphate (TPP; 100 µmol/l) on ^{3}H-FA apical uptake by NTB (white bars) and DTB (black bars) cells. Cells were incubated with 20 nmol/l ^{3}H-FA for 6 min at pH 5.5 in the presence of the compound (n = 6–11, from 2 to 4 distinct placentas) or the respective solvent (control; n = 5–13). Values shown are arithmetic means ± SEM. *Significantly different from control (P < 0.05); †significantly different from NTB cells (P < 0.05).

D-glucose, insulin, leptin, lipopolysaccharide (LPS), and TNF-α on the uptake of ^{3}H-FA by BeWo cells at physiological pH.

Hyperglycemia (10 and 30 mmol/l D-glucose (8)) and hyperinsulinemia (50 and 100 nmol/l insulin (15)) were devoid of effect on ^{3}H-FA uptake by BeWo cells compared with isosmotic normoglycemic (5.6 mmol/l glucose) and normoinsulinemic (10 pmol/l insulin (16)) conditions (Figure 4a,b).

On the other hand, as shown in Figure 4c, 100 ng/ml leptin (experimental hyperleptinemia (15), corresponding to ~3 times the plasma levels of leptin found in women with GDM) decreased ^{3}H-FA uptake by BeWo cells up to 25% in a time-dependent manner compared with leptin 1 ng/ml (which is in the range of concentrations found in normal pregnancies (17)). Curiously, a 4-h exposure of BeWo cells to 1, 100, 300, or 1,000 ng/ml leptin increased ^{3}H-FA uptake to 121.9 ± 4.7%; 114.3 ± 3.7%; 109.5 ± 2.8%; or 119.8 ± 8.6%, respectively (n = 11) compared with uptake in the total absence of this hormone.

Finally, a 4-h exposure of BeWo cells to 1 or 10 µg/ml LPS concentrations known to induce proinflammatory cytokine (interleukin-6 and TNF-α) secretion in trophoblast cells (18) and to 300 ng/l TNF-α itself significantly reduced ^{3}H-FA uptake (Figure 4d). Smaller (1 h) or longer (24 h) periods of exposure to these proinflammatory conditions (data not shown) or to lower concentrations of TNF-α (Figure 4d) did not alter ^{3}H-FA transport.

We next tested the effect of selected GDM conditions on the kinetic parameters of ^{3}H-FA uptake in BeWo cells. A 4-h and a 24-h treatment with 1 and 100 ng/ml leptin, respectively, and a 4-h treatment with 10 µg/ml LPS did not alter the K_m and V_{max} of ^{3}H-FA uptake by this cell line (data not shown).

Hyperleptinemia-Induced Inhibition of ^{3}H-FA Uptake Is Independent of Signaling through JAK/STAT, PI3K, PKA, PKC, and MAPK

The functions attributed to leptin depend on its binding to OB-R leptin receptors, which have been localized in the human syncytiotrophoblast (19), resulting in activation of the following signal transduction pathways: janus kinases (JAK)/signal transducers and activators of transcription (STAT), phosphoinositide 3-kinase (PI3K), protein kinases (PK) A and C and mitogen-activated protein kinases (MAPK) (extracellular-signal-regulated-kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK) (20).

Thus, we investigated the signaling mechanisms involved in the inhibitory effect mediated by leptin on ^{3}H-FA uptake by BeWo cells by assessing the effect of exposing BeWo cells for 24 h to inhibitors of intracellular signaling pathways, to leptin, or to both.

The role of JAK2/STAT3 in leptin (100 ng/ml)-induced inhibition of ^{3}H-FA uptake was investigated by treating BeWo cells for 24 h with 5 µmol/l of the well-established JAK2 inhibitor AG490 (21). Of note, the inhibitory effect of leptin on the uptake of ^{3}H-FA in the presence of AG490 was smaller than the inhibitory effect of leptin alone (Figure 5). However, western blotting phosphorylation assays failed to confirm the involvement of JAK2/STAT3 signaling in the leptin effect in BeWo cells (data not shown).

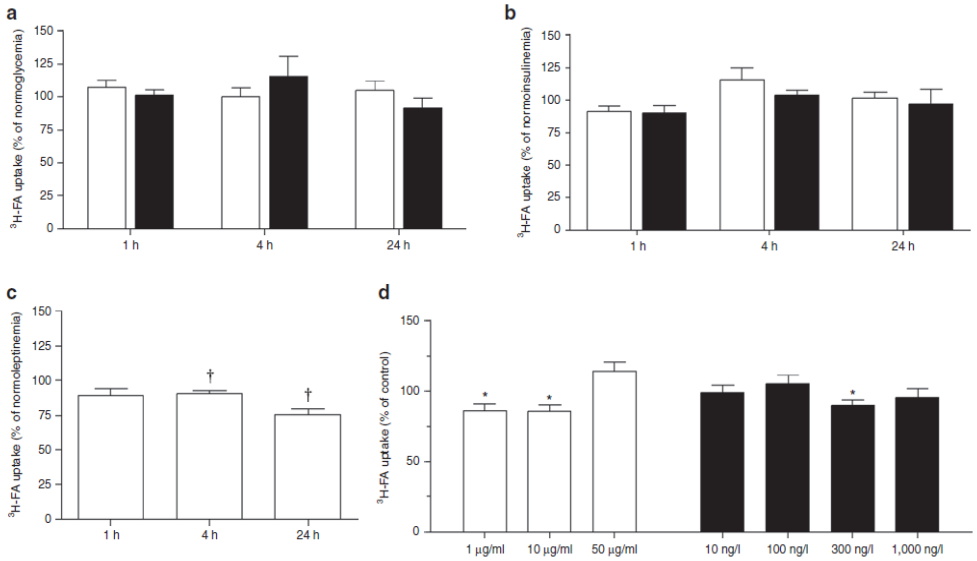

Figure 4. Effect of GDM-associated conditions on ^{3}H-FA apical uptake by BeWo cells. Cells were exposed to (**a**) D-glucose at 10 or 30 mmol/l (white and black bars respectively; $n = 8–9$) or 5.6 mmol/l (supplemented with mannitol up to 10 or 30 mmol/l for isosmotic control, corresponding to normoglycemia (100%), $n = 9$) for the indicated periods of time; (**b**) insulin at 1 or 50 nmol/l (white and black bars respectively; $n = 8–12$) or at 0.01 nmol/l (normoinsulinemia (100%), $n = 9–12$) for the indicated periods of time; (**c**) leptin at 100 ng/ml (white bars; $n = 8–10$) or 1 ng/ml (normoleptinemia (100%), $n = 9–11$) for the indicated periods of time; and (**d**) LPS 1–50 µg/ml (white bars $n = 12–17$), tumor necrosis factor-α at 10–1,000 ng/l (black bars; $n = 10–11$), or the respective solvents (control (100%); $n = 11–14$) for 4 h. Initial rates of uptake were determined in cells incubated with 50 nmol/l ^{3}H-FA for 6 min at pH 7.5. Values shown are arithmetic means ± SEM. *Significantly different from control ($P < 0.05$); †Significantly different from normoleptinemia ($P < 0.05$). FA, folic acid; GDM, gestational diabetes mellitus; LPS, lipopolysaccharide.

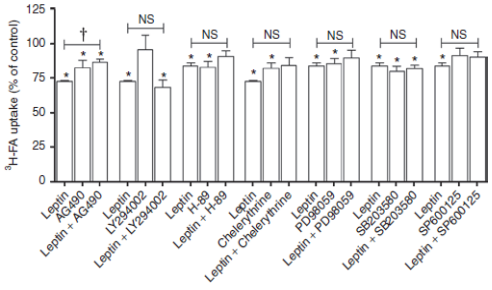

Figure 5. Effect of inhibitors of signaling pathways on the apical uptake of ^{3}H-FA and on leptin-induced inhibition of ^{3}H-FA uptake by BeWo cells. Initial rates of uptake were determined in cells incubated for 6 min with 50 nmol/l ^{3}H-FA after treatment for 24 h with 100 ng/ml leptin, 5 µmol/l AG490, 100 ng/ml leptin + 5 µmol/l AG490 (leptin + AG490), 1 µmol/l LY294002, 100 ng/ml leptin + 1 µmol/l LY294002 (leptin + LY294002), 1 µmol/l H-89, 100 ng/ml leptin + 1 µmol/l H-89 (leptin + H-89), 0.1 µmol/l chelerythrine, 100 ng/ml leptin + 0.1 µmol/l chelerythrine (leptin + chelerythrine), 2.5 µmol/l PD98059, 100 ng/ml leptin + 2.5 µmol/l PD98059 (leptin + PD98059), 9.6 µmol/l SB203580, 100 ng/ml leptin + 9.6 µmol/l SB203580 (leptin + SB203580), 5 µmol/l SP600125, and 100 ng/ml leptin + 5 µmol/l SP600125 (leptin + SP600125) ($n = 6–11$). Values shown are arithmetic means ± SEM. *Significantly different from control ($P < 0.05$); †significantly different from leptin ($P < 0.05$). FA, folic acid; NS, not significant.

With respect to the PI3K pathway, LY29400 (1 µmol/l), a specific inhibitor of PI3K activity (22), was not able to alter ^{3}H-FA uptake by itself and it did not affect the inhibitory effect of leptin on ^{3}H-FA uptake, indicating that PI3K signaling is not involved in ^{3}H-FA baseline uptake or in the leptin effect on this process (Figure 5).

H-89 (23) (1 µmol/l) and chelerythrine (24) (0.1 µmol/l), two specific inhibitors of PKA and PKC, respectively, reduced ^{3}H-FA uptake (by 17–18%), indicating that PKA and PKC activation is required for the baseline transport of this vitamin (Figure 5). However, the inhibitory effect of leptin on ^{3}H-FA uptake was not affected by either, excluding the involvement of PKA and PKC on the leptin inhibitory effect on ^{3}H-FA uptake by BeWo cells.

Finally, the involvement of MAPK pathways was investigated by testing the effect of specific inhibitors of MAPK/ERK kinase (MEK) (25) (PD980592.5 µmol/l), p38 MAPK (26) (SB2035809.6 µmol/l), and c-Jun N-terminal kinase (27) (SP600125 5 µmol/l). Uptake of ^{3}H-FA was reduced (by 14–20%) in the presence of either PD98059 or SB203580 but it was not affected by SP600125. Hence, MEK and p38 MAPK, but not c-Jun N-terminal kinase activation, appear to be required for ^{3}H-FA baseline transport into BeWo cells (Figure 5). Moreover, the inhibitory effect of leptin on ^{3}H-FA uptake was not affected by either of these compounds, thereby

excluding the involvement of the MAPK pathway in the inhibitory effect of leptin.

DISCUSSION

It is currently accepted that GDM precipitates offsprings' risk for developing cardiometabolic complications later in life (10,28). However, the exact molecular mechanisms underlying this programming effect are still unknown. One speculative explanation is that the intrauterine environment during GDM, perturbed by conditions such as hyperglycemia, hyperinsulinemia or even hyperleptinemia, and increased inflammatory environment, may induce epigenetic changes that will ultimately influence fetoplacental physiology and developmental programming (29).

Given that folates are obligatory cofactors for the provision of methyl groups for epigenetic regulation of gene expression and that little is known about the influence of GDM on fetoplacental folate homeostasis, the aims of this work were to investigate whether GDM affects FA placental transport and to identify specific GDM molecular hallmarks that may interfere with this process. To do this, we used two different approaches. First, we characterized ^{3}H-FA transport in human cytotrophoblasts isolated from normal and GDM pregnancies. Second, we investigated the effect of specific GDM conditions on ^{3}H-FA uptake in the BeWo cell line, a placental cellular model.

Both NTB and BeWo cells have been shown by us (30–32) and by others (33) to be suitable models for the study of placental transport mechanisms. Indeed, FA uptake characteristics and modulation have been shown to be very similar in both models (34).

When we compared ^{3}H-FA transport characteristics in NTB and DTB cells, we found that, although GDM does not significantly change the steady-state intracellular accumulation of ^{3}H-FA, DTB cells have higher rates of inward and outward transport, suggesting that a higher turnover of intracellular FA is required in these cells to maintain normal FA homeostasis.

The pH-dependence profiles of ^{3}H-FA uptake by NTB and DTB cells revealed an acidic optimum pH, pointing to the functional presence of the high-affinity folate: H^+ symporter PCFT. In addition, the greater pH-dependence observed in DTB cells for low pH values may indicate a higher PCFT:RFC1 relative activity in these cells. This finding is confirmed by the greater PTX-induced inhibition of uptake at pH 5.5 in DTB as compared with NTB cells (PTX is a high-affinity PCFT substrate but a very-low-affinity RFC1 substrate (35)).

Taken together, these results suggest that ^{3}H-FA uptake by DTB cells is more dependent on PCFT, although quantitatively similar, compared with NTB cells. Similarly, previous results from our group revealed that GDM modulates the interplay of different L-methionine transporters, although it does not quantitatively affect the amino acid transport capacity (data not shown). The strict clinical follow-up of GDM pregnant women may have masked potential functional differences in placental transport capacity of both nutrients.

To reinforce the biological significance of our findings, similar studies could be performed in other placental models such as placental perfusion or syncytiotrophoblast plasma membrane vesicles isolated from normal and GDM pregnancies.

Hyperglycemia, hyperinsulinemia, hyperleptinemia (12), and increased inflammation (13) are associated with GDM. To understand whether these molecular markers alter ^{3}H-FA placental uptake, we investigated their effects on ^{3}H-FA uptake by BeWo cells.

We verified that high concentrations of glucose and insulin did not affect ^{3}H-FA uptake. Accordingly, our group had previously demonstrated that ^{3}H-FA uptake by NTB cells was insensitive to short-term hyperglycemia and to both short- and long-term insulin exposure (32).

Of note, experimental hyperleptinemic conditions were revealed as an inhibitor of ^{3}H-FA uptake in a time-dependent manner when compared with uptake in the presence of physiological levels of leptin. In addition, high levels of LPS, known to induce proinflammatory cytokine TNF-α secretion in trophoblasts (18), or TNF-α itself inhibited ^{3}H-FA uptake by BeWo cells. Curiously, LPS and TNF-α inhibitory effects disappear for the highest concentrations tested. It is possible, as described by Torricelli *et al.* (18), that in our cell system, the highest concentrations of such proinflammatory stimuli generate a negative feedback loop by the locally inducing anti-inflammatory cytokine production that may attenuate proinflammatory-driven responses, such as inhibition of ^{3}H-FA uptake.

To our knowledge, this is the first time that an effect of leptin, LPS, and TNF-α on FA placental transport has been described. These findings support the idea advanced by others that leptin (19), and eventually TNF-α (36), may act as regulators of placental nutrient transport and therefore of fetal growth.

In this respect, Jansson *et al.* (15) and von Versen-Hoynck *et al.* (19) demonstrated that leptin increased system A amino acid transporter activity in placental villous fragments. In those works, leptin effect is expressed relative to the absence of the hormone. Of note, when we express the effect of 4 h leptin relative to the absence of this hormone, we also observe an increase in ^{3}H-FA uptake. However, when we express the effect of leptin relative to normal leptin concentrations in pregnancy, which is physiologically more relevant, we observe the above referred time-dependent reduction of ^{3}H-FA uptake. These observations indicate that the choice of control conditions for the study of pathology markers should take into account the levels of those markers in the healthy state.

Concerning TNF-α effects on placental nutrient transport, different findings have been reported. High levels of this cytokine have been shown to decrease L-methionine uptake (data not shown) or to increase arachidonic and docosahexaenoic acid uptake (data not shown) or system A activity (36) in human trophoblasts. Altogether, these observations reinforce the idea that TNF-α may well act as a regulator of placental nutrient transport.

The search for intracellular pathways that could be involved in the inhibitory effect of leptin on ^{3}H-FA uptake by BeWo showed that ^{3}H-FA uptake was partially inhibited by JAK2/STAT3, PKA, PKC, extracellular signal–regulated kinase/mitogen-activated protein kinase kinase, and p38 MAPK

pharmacological inhibition. Accordingly, folate transport in rat intestinal cells had already been described to be under the control of PKA, PKC (37).

In addition, pharmacological assays indicated a partial reversion of the leptin effect by the presence of AG490 (an inhibitor of JAK2 (21)), an effect that was not confirmed by western blotting assays. The failure in activation of JAK2/STAT3 signaling cascade by leptin had already been previously observed in BeWo cells by Caüzac *et al.* (38) who proposed that this pathway is not functional in this placental cell model.

The lack of involvement of PI3K and MAPK on leptin effect is in agreement with the lack of effect of insulin because PI3K and the MAPK phosphorylation cascades are in the cross talk of both leptin and insulin stimulation of placental cells (39).

In conclusion, our work demonstrates that GDM modulates ^{3}H-FA uptake by placental cells and that leptin, as well as TNF-α, downregulate it.

These conclusions are particularly interesting if we consider that leptin has early emerged as an important player in fetal programming (40) and may lead to the speculation that this programming effect could be related to leptin's observed effect on folate placental homeostasis.

METHODS

Materials

In this study, we used ^{3}H-FA ([3,5,7,9-^{3}H]-FA sodium salt; specific activity 40.0 Ci mmol^{-1}) (American Radiolabeled Chemicals, St. Louis, MO); 5-methyltetrahydrofolate disodium salt, amethopterin, chelerythrine chloride, Dulbecco's modified Eagle's medium, FA, H-89 dihydrochloride hydrate, Ham's F12K medium (Kaighn's modification), human recombinant insulin, human recombinant TNF-α, LPS from *Escherichia coli* 0111:B4, LY294002 hydrochloride, PD98059, SITS, SP600125, TPP, and tyrphostin AG490 (Sigma, St. Louis, MO); human recombinant leptin (Invitrogen, Carlsbad, CA); PTX (Eli Lilly, Indianapolis, IN); and SB203580 (Alomone Labs, Jerusalem, Israel).

Collection of Human Placentas

Collection and processing of human placentas were approved by the Ethics Committee for Health of C.H.S. João, Porto. Human placentas were obtained, with informed consent, at the Department of Obstetrics and Gynecology of C.H.S. João from uncomplicated or GDM term pregnancies (37–41 wk) within half an hour after spontaneous delivery or elective cesarean section. Control placentas represented normal pregnancies with no associated maternal or fetal pathology and were collected at random.

In pregnant women without prior known diabetes, the diagnosis of GDM was performed using a two-step approach. All pregnant women were tested by a 50-g glucose challenge test at 24–28 wk of gestation. In those with a blood glucose level ≥140 mg/dl (7.8 mmol/l), 1 h after the oral glucose load a diagnostic oral glucose tolerance test was performed. GDM was diagnosed according to the criteria defined by Carpenter and Coustan (41). These pregnancies were not associated with any major maternal or fetal pathology in addition to GDM. Women with GDM were treated with diet and exercise therapy up to the time of delivery. Insulin therapy was introduced whenever fasting blood glucose level was ≥5 mmol/l or 2-h postprandial blood glucose level was ≥6.7 mmol/l, despite consistent dietary and exercise adjustments. Clinical, anthropometric, and demographic data for control or GDM groups are given in Table 1.

Primary Culture of Human Cytotrophoblasts

Villous cytotrophoblasts corresponding to control or GDM pregnancies (NTB and DTB cells, respectively) were isolated using a modification of the technique described by Kliman *et al.* (42) as previously described by Keating *et al.* (31). Briefly, tissue was digested in Hank's balanced salt solution containing 0.15% trypsin (Invitrogen) and 0.02% DNAse I (Sigma) and the resulting cell suspensions were run in a discontinuous Percoll gradient (Sigma). Then, cytotrophoblast pellets were resuspended in Dulbecco's modified Eagle's medium and seeded on 24-well plastic cell culture clusters (2 cm^2; diameter 16 mm; TPP, Trasadingen, Switzerland) at a density of 6–7.5 × 10^5 cells/cm^2. After 72 h in culture, cells aggregated to form syncytial clumps corresponding to syncytiotrophoblasts and were used for transport experiments.

BeWo Cell Culture

The BeWo cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (ACC-458; DMSZ, Braunschweig, Germany) and was used between passage numbers 6 and 29. The cells were cultured as previously described (31).

FA Uptake Studies

The transport experiments in NTB, DTB, and BeWo cells were performed in buffer with the following composition (in mmol/l): 125 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 12.5 HEPES–NaOH, 12.5 2-[N-morpholino]ethanesulfonic acid hydrate (Sigma), 1.2 $MgSO_4$, 1.2 $CaCl_2$, and 5.6 mmol/l D(+)-glucose (Merck, Darmstadt, Germany), pH 5.5 or 7.5 (unless otherwise stated), as previously described (32). ^{3}H-FA was used in concentrations of 20 or 50 nmol/l (except in the experiments for determination of the kinetics of ^{3}H-FA uptake).

The concentrations of folate analogs of SITS and of TPP used in the characterization of FA uptake were chosen based on previous work from our group (30,32,43) and others (7,35,44).

The total protein content of cell monolayers was determined as described by Bradford (45), and it was not altered by any of the compounds tested, indicating that cell viability was not compromised (data not shown).

Effect of GDM-Associated Hallmarks on ^{3}H-FA Uptake in BeWo Cells

The effect of GDM-associated conditions on ^{3}H-FA uptake was tested at physiological pH in 5- to 8-d-old BeWo cell cultures. Cells were exposed to different concentrations of D-glucose, insulin, leptin, LPS or TNF-α, or the respective solvent in fetal calf serum–free culture medium for 1, 4, or 24 h. After these treatments, transport experiments were performed as previously described (32). The effect of inhibitors of signaling pathways was tested by cultivating cells, during specific time periods, in fetal calf serum–free medium containing selected GDM markers (which significantly altered ^{3}H-FA uptake) plus these inhibitors or the respective solvents.

Calculations and Statistics

For the analysis of the time course of ^{3}H-FA uptake, the parameters of equation (1) were fitted to the experimental data using a nonlinear regression analysis (46).

$$A(t) = k_{in}/k_{out}(1 - e^{-k_{out}\cdot t}) \quad (1)$$

$A(t)$ represents the accumulation of ^{3}H-FA at time t, k_{in} and k_{out} are the rate constants for inward and outward transport, respectively, and t is the incubation time. A_{max} is defined as the accumulation at steady state ($t \to \infty$). k_{in} is given in pmol·mg protein^{-1}·min^{-1} and k_{out} in min^{-1}. Arithmetic means are given with SEM.

For the analysis of the saturation curve of ^{3}H-FA uptake, the parameters of the Michaelis–Menten equation were fitted to the experimental data using nonlinear regression analysis (46).

Statistical significance of the difference between various groups was evaluated by one-way or two-way (in the case of pH-dependence) ANOVA test followed by the Bonferroni test. For comparison between two groups, Student's t test was used. Differences were considered significant when $P < 0.05$.

The value of n indicates the number of replicates of at least two different experiments or placentas.

ACKNOWLEDGMENTS

We thank Joana Silva and Paula Soares (Institute of Molecular Pathology and Immunology, University of Porto, Portugal) for the kind donation of the STAT3 and phospho-STAT3 antibodies.

STATEMENT OF FINANCIAL SUPPORT

This work was supported by Fundação para a Ciência e Tecnologia (FCT) and Programa Operacional Temático de Fatores de Competitividade (COMPETE), Quadro de Referência Estratégico Nacional (QREN), and Fundo Europeu de Desenvolvimento Regional (FEDER) (PTDC/SAU-OSM/102239/2008; SFRH/BPD/40170/2007; and SFRH/BD/63086/2009).

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Original Article

Reproductive Sciences
20(12) 1492-1507

DOI: 10.1177/1933719113488442
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L-Methionine Placental Uptake: Characterization and Modulation in Gestational Diabetes Mellitus

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Abstract

Our aim was to investigate the influence of gestational diabetes mellitus (GDM) and GDM-associated conditions upon the placental uptake of ^{14}C-L-methionine (^{14}C-L-Met). The ^{14}C-L-Met uptake by human trophoblasts (TBs) obtained from normal pregnancies (normal trophoblast [NTB] cells) is mainly system L-type amino acid transporter 1 (LAT1 [L])-mediated, although a small contribution of system y^+LAT2 is also present. Comparison of ^{14}C-L-Met uptake by NTB and by human TBs obtained from GDM pregnancies (diabetic trophoblast [DTB] cells) reveals similar kinetics, but a contribution of systems A, LAT2, and b^{0+} and a greater contribution of system y^+LAT1 appears to exist in DTB cells. Short-term exposure to insulin and long-term exposure to high glucose, tumor necrosis factor-α, and leptin decrease ^{14}C-L-Met uptake in a human TB (Bewo) cell line. The effect of leptin was dependent upon phosphoinositide 3-kinase, extracellular-signal-regulated kinase 1/2 (ERK/MEK 1/2), and p38 mitogen-activated protein kinase. In conclusion, GDM does not quantitatively alter ^{14}C-L-Met placental uptake, although it changes the nature of transporters involved in that process.

Keywords

gestational diabetes, placenta, transport, L-methionine

Introduction

The placenta is the main interface between the maternal and the fetal blood circulations, being responsible for the transfer of nutrients from mother to fetus and clearance of waste metabolites from fetal blood.[1] This function is mediated by transporters present both at the maternal-facing microvillous membrane and at the fetal-facing basal membrane of the syncytiotrophoblast (STB), the polarized epithelium that constitutes the functional unit of the placenta. The activity of these transporters will largely determine the extent to which the compounds will cross the placenta and enter the fetal blood circulation.[2] Changes in placental nutrient transfer capacity will, therefore, have important consequences for the growth and development of the fetus.[1]

Methionine (L-Met) is a nutritionally essential large neutral amino acid indispensable to the fetus. The L-Met is required not only for fetal protein synthesis and as an energetic substrate for fetal oxidative catabolism but also for the production of S-adenosyl methionine that is the principal methyl group donor in mammalian cells, being thus essential for methylation reactions.[3] The importance of L-Met in fetal development is well demonstrated by the occurrence of pregnancies affected by neural tube defects in women with low dietary intake of L-Met.[4]

Transport of amino acids across the human placenta is a complex process, resulting in amino acid concentrations in the fetal blood circulation substantially higher than those in maternal plasma. The STB expresses at least 15 different amino acid transporters, each mediating the active uptake of several different amino acids and each specific amino acid being able to be transported by several distinct transport systems.[5,6]

Gestational diabetes mellitus (GDM), defined as a degree of glucose intolerance with the onset or first recognition during pregnancy (usually toward the late-second and early third trimesters), affects about 7% of all pregnancies.[7] Hyperglycemia, hyperinsulinemia, and insulin resistance are the hallmarks of

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this disease and also of type 2 diabetes.[7] Although not exclusive to GDM or type 2 diabetes, hyperleptinemia[8] and elevated plasma levels of proinflammatory markers[9] are also associated with these closely related diseases.

The most common perinatal complication associated with GDM is fetal macrosomia, which is a risk factor for operative delivery and traumatic birth injury.[10] Furthermore, GDM presents fetal programming effects, because there is an increased risk for the offspring to develop some cardiovascular and metabolic diseases (obesity, type 2 diabetes mellitus, and hypertension) later in life.[11,12] The GDM is also associated with adverse health outcomes for the mother, including type 2 diabetes mellitus and metabolic syndrome, later in life.[12] The mechanisms whereby GDM increases the risk of fetal overgrowth and development of metabolic diseases later in life are still unclear but are likely to involve changes in nutrient supply to the fetus[13] and placental development and blood flow.[3]

Epigenetic regulation, in particular gene methylation and histone modification of fetal and placental genome, plays a crucial role in gene expression, imprinting processes, and embryonic development, thereby programming the fetus for future development of diseases.[3] Biological methylation reactions are dependent on the availability of amino acids such as L-Met (see above) and cofactors such as folates, vitamin B_{12}, and choline.[14] So, changes in placental transport of these compounds will alter the availability of these methyl donors to the fetus, providing a direct link between placental function, gene methylation, and fetal programming.[3] Interestingly enough, GDM has been associated with specific changes in nutrient transporters[15] and particularly, amino acid transporters. However, knowledge on the placental transport of amino acids in GDM remains scarcely studied in vitro, and the data available are quite conflicting.[16-19] In addition, despite the data provided by human studies,[20,21] the mechanism responsible for L-Met placental uptake in normal pregnancies is still not completely understood.

Because GDM may have programming effects and because long-term effects of certain stimuli during pregnancy may be caused by genome methylation, we hypothesize that GDM may interfere with the placental transport of the methyl group carrier L-Met. For this purpose, we first determined the characteristics of L-Met uptake by normal human trophoblasts (TBs) using 2 cellular models: primary cultured human cytotrophoblasts (TB cells) obtained from normal pregnancies (normal trophoblast [NTB] cells) and the Bewo choriocarcionoma cell line. The TB cells are considered as a suitable model to study the placental transport function,[22,23] because they spontaneously differentiate into a functional and polarized STB-like structure that retains all the cellular machinery of the in vivo STB.[23] Then, the influence of GDM and specific GDM-associated conditions upon this process was investigated. For this, a comparison between L-Met uptake in NTB cells and in cytotrophoblasts isolated from GDM pregnancies (diabetic trophoblast [DTB] cells) was made, and an investigation of the effects of elevated levels of glucose, insulin, leptin, and proinflammatory mediators (lypopolissacharide [LPS] and tumor necrosis factor-α [TNF-α]) in Bewo cells was performed.

Materials and Methods

Reagents

The reagents used include ^{14}C-L-methionine (^{14}C-L-Met; specific activity 40-60 mCi/mmol; American Radiolabeled Chemicals, St Louis, MO), L-alanine, antibiotic/antimycotic solution (100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B), L-arginine monohydrochloride, 2-amino-2-norbornanecarboxylic acid (BCH), bovine serum albumin (BSA), chelerythrine chloride, collagen type I from rat tail, Dulbecco modified Eagle medium (DMEM), DNAse I (deoxyribonuclease I from bovine pancreas), fetal calf serum (FCS), Ham F12K medium (Nutrient Mixture F12-Ham Kaighn modification), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), human insulin (recombinant, expressed in yeast), human TNF-α (recombinant, expressed in HEK293 cells), H-89 dihydrochoride hydrate, lipopolysaccharides from *Escherichia coli* 0111: B4, L-lysine monohydrochloride, LY-294002 hydrochloride, D-leucine, α-(methylamino)isobutyric acid (MeAIB), 2-[N-morpholino]ethanesulfonic acid (MES) hydrate), PD 98059, Percoll, L-phenylalanine, D-phenylalanine, L-serine, SP 600125, L-tryptophan, trypsin-EDTA solution, tyrphostin AG 490 (Sigma, St Louis, MO), dimethylsulfoxide (DMSO), D(+)-glucose, Tris(tris-(hydroxymethyl)-aminomethane hydrochloride), Triton X-100 (Merck, Darmstadt, Germany), Hank balanced salt solution (HBSS), trypsin 2.5% (×10 solution, GIBCO; Invitrogen Corporation, Carlsbad, California), recombinant human leptin (Invitrogen Corporation), D-mannitol (Difco Laboratories, Detroit, MI), rapamycin (from *Streptomyces hygroscopicus*), and SB 203580 (Alomone Labs Ltd, Jerusalem, Israel), and Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany).

The drugs to be tested were dissolved in water, DMSO, HCl 0.01 mol/L, 0.1% (w/v) BSA, or 0.1% (w/v) BSA in phosphate-buffered saline. The final concentration of these solvents in the buffer and culture medium was 1% (v/v). Controls for the drugs were run in the presence of the solvent. Neither of the solvents had a significant effect on ^{14}C-L-Met uptake (results not shown).

Collection of Human Placenta

Collection and processing of human placenta were approved by the ethical committee of Centro Hospitalar S. João (Porto, Portugal). Human placenta were obtained at the Department of Obstetrics and Gynecology of Centro Hospitalar S. João from uncomplicated (control, n = 14) and GDM (n = 9) singleton term pregnancies (37-41 weeks), within half an hour after spontaneous delivery or elective cesarean section. Control placenta represented normal pregnancies with no associated maternal or fetal pathology and were collected at random.

In pregnant woman without prior known diabetes, the diagnosis of GDM was performed by a 2-step approach. All pregnant women were tested by a 50-g glucose challenge test at 24 to 28 weeks of gestation. In those with a blood glucose level ≥140 mg/dL (7.8 mmol/L) 1 hour after the oral glucose load, a diagnostic oral glucose tolerance test (OGTT) was performed.

Table 1. Clinical, Anthropometrical, and Demographic Data of the 2 Study Groups.[a]

	Control	GDM
Mothers		
n	14	9
Maternal age, years	32.6 ± 1.3	33.8 ± 1.2
BMI before delivery,[b] kg/m^2	29.1 ± 1.9	32.6 ± 0.8
Gravida (n)	2.2 ± 0.4	2.3 ± 0.3
Parity (n)	0.9 ± 0.3	1.2 ± 0.3
Mode of delivery		
Vaginal, n (%)	5 (36)	4 (44)
Cesarean, n (%)	9 (64)	5 (56)
Therapeutics of GDM, n (%)	-	Nutritional: 5 (56) Insulin: 4 (44)
Fasting blood glucose, mmol/L[c]		
All	4.0 ± 0.1	4.5 ± 0.3[d]
GDM without insulin therapy		4.4 ± 0.4
GDM with insulin therapy		4.8 ± 0.3[d]
HbA_{1C}, %[e]		
All	-	5.4 ± 0.2
GDM without insulin therapy		5.2 ± 0.1
GDM with insulin therapy		5.7 ± 0.3
Periconceptional FA use, n (%)[f]	12 (86)[g]	6 (67)[h]
Smokers, n (%)	0 (0)[i]	0 (0)
Infants		
Gestational age at birth, weeks[j]	39.4 ± 0.3	39.1 ± 0.3
Birth weight, g[k]	3226 ± 107	3383 ± 177
Length, cm[l]	48.2 ± 0.4	49.4 ± 0.5
SGA newborn, n (%)[m]	1 (7)	0 (0)
AGA newborn, n (%)	11 (79)	7 (78)
LGA newborn, n (%)	2 (14)	2 (22)
Placental weight, g	594.4 ± 32.2	686.6 ± 47.6
Gender, n (%)	Male: 3 (21) Female: 11 (79)	Male: 3 (33) Female: 6 (67)
5-Minute Apgar score	9.3 ± 0.2	9.1 ± 0.2

Abbreviations: AGA, adequate for gestational age; BMI, body mass index; FA, folic acid; GDM, gestational diabetes mellitus; HbA_{1c}, hemoglobin A_{1c}; LGA, large for gestational age; SEM, standard error of the mean; SGA, small for gestational age.

[a]Values represent mean ± SEM.

[b]Parameter unknown for 2 patients (one from each group).

[c]Values obtained at 24 to 28 weeks of gestation.

[d]Significantly different from control ($P < .05$).

[e]Values obtained at 35 to 36 weeks of gestation. Parameter unknown for all the patients from control group.

[f]Dosage and initiation period unknown.

[g]Parameter unknown for 2 patients.

[h]Parameter unknown for 3 patients.

[i]Parameter unknown for 2 patients.

[j]Gestational age: number of completed weeks at the time of delivery, determined by prenatal ultrasound at 11 to 13 weeks.

[k]Birth weight was evaluated to the nearest gram.

[l]Length was evaluated to the nearest tenth of a centimeter after birth.

[m]Classified according to the published reference standards.[25]

The GDM was diagnosed when 2 or more of the following plasma glucose concentrations were met or exceeded, according to the criteria defined by Carpenter and Coustan[24]: fasting blood glucose level ≥ 95 mg/dL (5.3 mmol/L) and/or blood glucose level ≥ 180 mg/dL (10 mmol/L), 155 mg/dL (8.6 mmol/L), or 140 mg/dL (7.8 mmol/L) 1, 2, or 3 hours after a 100 g OGTT, respectively. These pregnancies were not associated with any major maternal or fetal pathology in addition to GDM. Women with diagnosed GDM were surveilled in Centro Hospitalar S. João and treated with diet and exercise therapy during the course of pregnancy up to the time of delivery. In 4 patients, insulin therapy was necessary. The criterion for initiating insulin therapy was the presence of a fasting blood glucose level ≥ 90 mg/dL (5 mmol/L) or a 2-hour postprandial blood glucose level ≥ 120 mg/dL (6.7 mmol/L), despite consistent dietary and exercise adjustments. Selected clinical, anthropometric, and demographic data for control or GDM groups are given in Table 1.

Primary Culture of Human Cytotrophoblasts (TB cells)

Villous TB cells obtained from control and GDM pregnancies (NTB and DTB cells, respectively) were isolated as described previously.[22] Briefly, fetal membranes and maternal decidua were removed, and villous tissue without macroscopic degenerative alterations present immediately below the umbilical cord insertion was cut and scraped from the blood vessels. The tissue was then digested in HBSS containing 0.15% trypsin and 0.02% DNAse I, and the resulting cell suspension was run in a discontinuous Percoll gradient. Then, cytotrophoblast pellets were collected and resuspended in DMEM/F-12 medium (containing 10% FCS and 1% antibiotic/antimycotic solution) and seeded on 24-well plastic cell culture clusters (2 cm^2; diameter 16 mm; Techno Plastic Products [TPP], Trasadingen, Switzerland) at a density of 6 to 7.5 × 10^5 cells/cm^2. After 72 hours in culture, the TB cells aggregate to form syncytial clumps corresponding to STBs and were then used for transport experiments.

To evaluate the purity of TB cell cultures, cells in chamber slides were fixed with 4% paraformaldehyde and immunolabeled with mouse antivimentin (BD Biosciences, San Jose, California) and anticytokeratin (Dako, Glostrup, Denmark) antibodies. Staining was performed with horseradish peroxidase-secondary antibody using DAB substrate kit, according to the manufacturer's instructions. Corresponding to epithelial TB cells, 95% of the cells were cytokeratin positive and less than 5% were vimentin positive, corresponding to fibroblast cells.

Bewo Cell Culture

The Bewo cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ GmbH, ACC-458) and was used between passage numbers 4 to 28. The cells were maintained in a humidified atmosphere of 5% CO_2 to 95% air and were grown in Ham F12K medium containing 2.5 g/L sodium bicarbonate, 15% (v/v) heat-inactivated FCS, and 1% (v/v) antibiotic/antimycotic solution. Culture medium was changed every 2 to 3 days, and the culture was passaged every 7 to 8 days. For subculturing, the cells were removed enzymatically (0.25% [v/v] trypsin-EDTA, 5 minutes, 37°C), passaged 1:3, and subcultured in plastic culture dishes (21 cm^2; diameter 60 mm; BD Falcon, New Jersey). For the experiments, Bewo

cells were seeded on collagen-coated 24-well plastic cell culture clusters (2 cm^2; diameter 16 mm; TPP). After 7 to 8 days in culture (90%-100% confluence), the cells were used in uptake experiments. At this moment, each cm^2 contained about 60 µg cell protein.

^{14}C-L-Met Uptake Studies in TB and Bewo Cells

The transport experiments were performed in buffer with the following composition (in mmol/L): 125 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 12.5 HEPES-NaOH, 12.5 MES, 1.2 $MgSO_4$, 1.2 $CaCl_2$, and 5.6 D(+)-glucose, pH 7.5. Initially, the culture medium was aspirated, and the cells were washed with 0.3 mL buffer at 37°C; then, the cell monolayers were preincubated for 20 minutes with 0.3 mL buffer at 37°C. Uptake was initiated by the addition of 0.2 mL buffer at 37°C containing 250 nmol/L ^{14}C-L-Met (except in the experiments for the determination of the kinetics of ^{14}C-L-Met uptake, as indicated). Incubation was stopped after 6 minutes (unless otherwise stated) by removing the incubation medium, rinsing the cells with 0.3 mL ice-cold buffer, and placing the cells on ice. The cells were then solubilized with 0.3 mL of 0.1% (v/v) Triton X-100 (in 5 mmol/L Tris–HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting and normalized for total cell protein. Total cell protein was determined by the Bradford method[25] using BSA as standard.

Pharmacological characterization of ^{14}C-L-Met uptake. Drugs to be tested were present during both the preincubation and the incubation periods (in a total of 26 minutes). Controls were run in the presence of the respective solvents.

Sodium dependence of ^{14}C-L-Met uptake. To study the influence of external Na^+ on the uptake of ^{14}C-L-Met, the cells were washed, preincubated, and incubated in NaCl-free buffer, NaCl (corresponding to 125 mmol/L) being isotonically replaced with either lithium chloride (LiCl) or choline chloride (ChCl).

Effect of GDM-Associated Conditions Upon ^{14}C-L-Met Uptake by Bewo Cells

The effect of some specific GDM-associated conditions upon ^{14}C-L-Met uptake was tested in Bewo cells. The cells were exposed to different concentrations of glucose, insulin, leptin, TNF-α, and LPS (or the respective solvent) in the culture media (without FCS) for 1, 4, 24, 48, 72, or 96 hours. In the 48-, 72-, and 96-hour exposure periods, the medium was renewed daily. After these treatments, transport experiments were performed. These experiments were identical to the ones described in the "^{14}C-L-Met uptake studies in TB and Bewo cells," section except that there was no preincubation period. So, the cells were incubated in buffer for 6 minutes in the presence of GDM conditions or the respective solvent. For 10 and 30 mmol/L glucose experiments, an isosmotic control was run using mannitol. None of the GDM conditions tested altered the Bewo cell viability, with the exception of 10 mmol/L D-glucose (72 hours), which increased it by 23% (results not shown).

In some experiments, we assessed whether the effect of GDM-associated conditions upon uptake of ^{14}C-L-Met in FCS-free culture media (which contain elevated amino acids concentrations[2,26]) and buffer would be similar, by choosing insulin as a paradigm.

The effect of BCH (a classical substrate of the system L transporter of amino acids[27]) upon ^{14}C-L-Met uptake under GDM conditions was also tested by exposing the cells to GDM-associated conditions or the respective solvents (as described above), and then preincubating (20 minutes) and incubating the cells (6 minutes) with ^{14}C-L-Met, in the absence or presence of BCH.

In some other experiments, the effect of inhibitors of intracellular signaling pathways on ^{14}C-L-Met uptake under specific GDM conditions was tested. In these studies, compounds (or the respective solvents) were present throughout the experiment, simultaneously with the GDM-associated conditions.

RNA Extraction and Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from NTB and DTB cells using the Tripure isolation reagent, according to the manufacturer's instructions (Roche Diagnostics).

Before complementary DNA (cDNA) synthesis, total RNA was treated with DNase I (Ambion Inc, Texas) to eliminate the potential genomic DNA contamination. Then, total RNA quantity and quality were assessed spectrophotometrically by measuring the absorbance ratio at 260:280 nm. In our RNA samples, this ratio was between 1.96 and 2.17. Resulting 2 µg of DNA-free RNA was reverse transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen Corporation) in 80 µL of final reaction volume, according to the manufacturer's instructions. Resulting cDNA was treated with RNase H (Invitrogen Corporation) to degrade unreacted RNA. For the quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), 2 µL of the 80 µL reverse transcription reaction mixture was used.

For the calibration curve, placental standard cDNA (using total RNA from NTB cells) was diluted in 5 different concentrations. The qRT-PCR was carried out using a LightCycler (Roche, Nutley, New Jersey). The 20 µL of reactions were set up in the microcapillary tubes using 0.5 µmol/L of each primer and 4 µL of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I; Roche). Cycling conditions were as follows: denaturation (95°C for 5 minutes), amplification, and quantification (95°C for 10 seconds, annealing temperature [AT] for 10 seconds, and 72°C for 10 seconds, with a single-fluorescence measurement at the end of the 72°C for 10 seconds segment) repeated 55 times, a melting curve program ([AT + 10)°C for 15 seconds and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement), and a cooling step to 40°C for 30 seconds. The ATs and sequence of primers are indicated in Table 2. The primer pair for β-actin

Table 2. Primer Sequences and ATs Used for Real-Time RT-PCR.

Gene Name	Primer Sequence (5′-3′)	AT, °C
β-Actin	Fwd: AGA GCC TCG CCT TTG CCG AT Rev: CCA TCA CGC CCT GGT GCC T	65
LAT2	Fwd: TCG CTG TGA CTT TTG GAG A Rev: GCC GAG AGG TGA AGA GA	64
SNAT1	Fwd: ACT ACC CTC TGC CAT AAA Rev: TAT AGC CAA GAT ACC CTA AGT	60
SNAT2	Fwd: GTC ATT GGT GGT CAT TCT T Rev: GTG GTG TTT ATT GTT TCG TTA	60
y^+LAT1	Fwd: AAC TGT GCC AGG GAC ACT Rev: GAG AAG AGG GCA GAG TAG AGG	65

Abbreviations: LAT2, L-type amino acid transporter 2; SNAT1, sodium-coupled neutral amino acid transporter 1; SNAT2, sodium-coupled neutral amino acid transporter 2; y^+LAT1, y^+L-amino acid transporter 1; Fwd, forward; Rev, reverse; RT-PCR, reverse transcription-polymerase chain reaction; AT, annealing temperature.

was kindly donated by Dr Joana Marques (Department of Genetics, Faculty of Medicine, University of Porto, Portugal). Each sample was tested in duplicate. For each gene, the mean threshold cycle was 19.06 to 28.42, and the intrassay coefficient of variation was 0.17% to 2.00%. Data were analyzed using LightCycler 4.05 analysis software (Roche). The amount of messenger RNA (mRNA) of each studied gene was normalized to the amount of mRNA of the housekeeping gene (β-actin). There was no effect of GDM on the expression levels of β-actin (results not shown).

Calculations and Statistics

For the analysis of the time course of ^{14}C-L-Met uptake, the parameters of the equation $A(t) = k_{in}/k_{out}(1 - e^{-k_{out}t})$ were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method.[28] $A(t)$ represents the accumulation of ^{14}C-L-Met at time t, k_{in} and k_{out} the rate constants for inward and outward transport, respectively, t the incubation time, and A_{max} the accumulation at steady state ($t \rightarrow \infty$).

For the analysis of the saturation curve of ^{14}C-L-Met uptake, the parameters of the Michaelis–Menten equation were fitted to the experimental data using a nonlinear regression analysis, using a computer-assisted method.[28]

Arithmetic means are given with standard error of the mean. Statistical significance of the difference between various groups was evaluated by 1-way analysis of variance test followed by the Bonferroni post test. For comparison between 2 groups, the Student t test was used. Differences were considered to be significant when $P < .05$.

The value of n indicates the number of replicates for at least 2 different experiments (Bewo cells) or placenta (TB cells).

Results

Clinical, Anthropometrical, and Demographic Characteristics of the Study Groups

As shown in Table 1, control and GDM groups were closely matched in terms of clinical, anthropometrical, and demographic data. The only difference between these 2 groups was maternal fasting blood glucose levels (which were determined at the time of GDM diagnosis [24-28 weeks of gestation]) that were significantly higher in the GDM group. Women with GDM having higher fasting blood glucose levels (Table 1) were subsequently treated with insulin. Insulin therapy was able to induce a good glycemic and metabolic control, as glycosylated hemoglobin A_{1c} levels were similar and fell within the acceptable range for managed diabetes (≤5.7%) in both insulin-treated and nontreated women with GDM near the end of pregnancy (35-36 weeks of gestation) .[29,30] Additionally, maternal weight gain (8.9 ± 3.4 and 6.6 ± 3.2 kg) and body mass index before delivery (31.6 ± 1.3 and 33.5 ± 0.9 kg/m^2), newborn weight (3433 ± 249 and 3344 ± 274 g) and length (41.1 ± 0.8 and 49.7 ± 0.8 cm), and placental weight (705 ± 98 and 673 ± 48 g) and gestational age at delivery (38.9 ± 0.3 and 39.3 ± 0.5 weeks) were all similar in both insulin-treated and nontreated women with GDM. All together, these data support that the GDM population in this study is homogenous, independent of insulin therapy, having similar glycemic and metabolic control after GDM diagnosis until the end of pregnancy. Newborn and placenta weights in the GDM group tended to be higher than in control group, but this difference did not reach statistical significance.

Characterization of ^{14}C-L-Met Uptake in NTB and DTB Cells

In a first series of experiments, we characterized and compared ^{14}C-L-Met uptake in NTB and DTB cells in terms of time and Na^+ dependence, kinetic parameters, and specificity of the carrier systems involved.

Time course. In these initial experiments, we determined the time course of accumulation of ^{14}C-L-Met in NTB and DTB cells. As shown in Figure 1A, both NTB and DTB cells accumulated ^{14}C-L-Met in a time-dependent way, uptake being linear for the first 6 minutes of incubation. On the basis of this information, subsequent experiments to characterize the uptake of this amino acid were performed using a 6-minute incubation time.

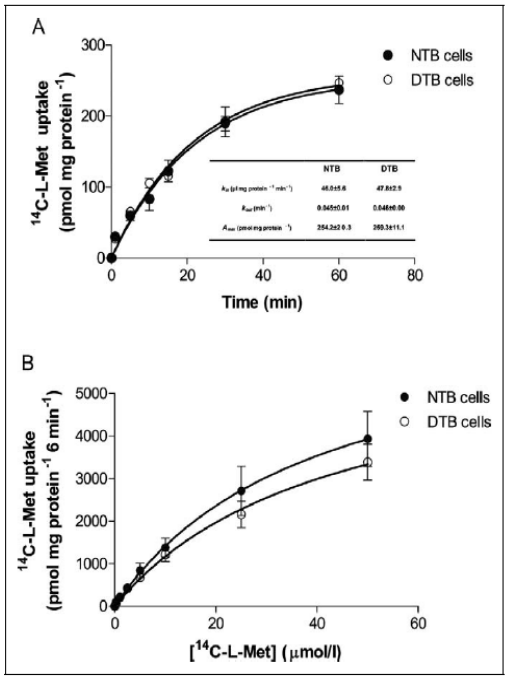

Figure 1. Time course (A) and kinetics (B) of ^{14}C-L-methionine (^{14}C-L-Met) uptake by normal trophoblast (NTB) and diabetic trophoblast (DTB) cells. For time course experiments, cells were incubated for various periods of time at 37°C with 250 nmol/L ^{14}C-L-Met, pH 7.5 (n = 6-7, from 3 distinct placenta). For kinetic experiments, initial rates of uptake were determined in cells incubated at 37°C with increasing concentrations of ^{14}C-L-Met (0.25-50 µmol/L) for 6 minutes (n = 9-12, from 3 to 4 distinct placenta). Shown is arithmetic mean ± standard error of the mean.

Analysis of the time course allowed the determination of the rate constant of inward transport (k_{in}), the rate constant of outward transport (k_{out}), and the steady state accumulation (A_{max}) of ^{14}C-L-Met, which were similar in NTB and DTB cells (Figure 1A).

Kinetics. In this set of experiments, the initial rates of ^{14}C-L-Met uptake at increasing substrate concentrations in the apical medium (from 0.25 to 50 µmol/L) were determined in NTB and DTB cells (Figure 1B). The evaluated kinetic parameters, K_m and V_{max}, were not different between NTB and DTB cells (K_m = 39.7 ± 20.4 and 43.0 ± 16.7 µmol/L for NTB and DTB cells, respectively [n = 9-12] and V_{max} = 7.04 ± 1.99 and 6.19 ± 1.36 nmol mg/prot/6 min for NTB and DTB cells, respectively [n = 9-12]).

Na^+ dependence. Different groups of transport systems for large neutral amino acids are present in both the microvillous and the basal plasma membranes of the STB. These comprise Na^+-dependent (eg, systems A and y^+L/y^+L-type amino acid transporter [LAT]) and Na^+-independent (eg, systems L and b^{0+}) transport systems.[6] So, we examined the effect of isosmotically replacing NaCl in the preincubation and incubation buffer with another monovalent cation (Li^+ or Ch^+) on ^{14}C-L-Met uptake by the NTB and DTB cells. Uptake was found to be partially Na^+ dependent in both NTB and DTB cells, as substitution of Na^+ by Li^+ or Ch^+ decreased it by ± 25% (Figure 2).

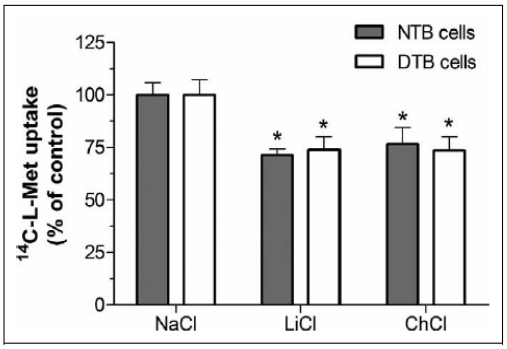

Figure 2. Extracellular Na^+ dependence of ^{14}C-L-methionine (^{14}C-L-Met) uptake in normal trophoblast (NTB) and diabetic trophoblast (DTB) cells incubated at 37°C with 250 nmol/L ^{14}C-L-Met for 6 minutes, at pH 7.5. NaCl in the preincubation and incubation buffer was isotonically replaced by either LiCl or choline chloride (ChCl) (n = 6-11, from 2 to 3 distinct placenta). Shown is arithmetic mean ± standard error of the mean. *Significantly different from control (NaCl; $P < .05$).

Pharmacological characterization. The specificity of the carrier system responsible for ^{14}C-L-Met uptake in NTB and DTB cells was investigated by determining the effect of a variety of unlabeled amino acids upon ^{14}C-L-Met transport. The amino acids tested were (1) 3 large neutral amino acids (BCH, a nonmetabolizable amino acid analogue,[27] L-Phe, and L-Trp[31]), which are substrates of LAT system, (2) the large neutral amino acids D-Leu and D-Phe, which are substrates of LAT1,[31] (3) the small neutral amino acids L-Ala[31,32] and L-Ser,[32] which are substrates of LAT2, (4) the cationic amino acids L-Arg and L-Lys,[33] which are substrates of y^+L and b^{0+} amino acid transporter systems, and L-Ala, which is also a substrate of y^+LAT2 but not of y^+LAT1,[6] and (5) the nonmetabolizable N-methylated amino acid analog MeAIB, a known substrate of system A.[27] Despite having similar substrate specificity, system y^+L and system b^{0+} transport neutral amino acids in the presence and absence of Na^+, respectively.[33]

Pharmacological characterization of ^{14}C-L-Met uptake in NTB and DTB cells revealed some overlapping characteristics. Namely, transport in both NTB and DTB cells was strongly reduced (by 40%-60%) by system L substrates BCH, L-Phe ,and L-Trp (Figure 3A), less markedly inhibited (by 30%) by D-Leu and only slightly inhibited (by 17%) by L-Ala (Figure 3B). However, distinct characteristics of ^{14}C-L-Met

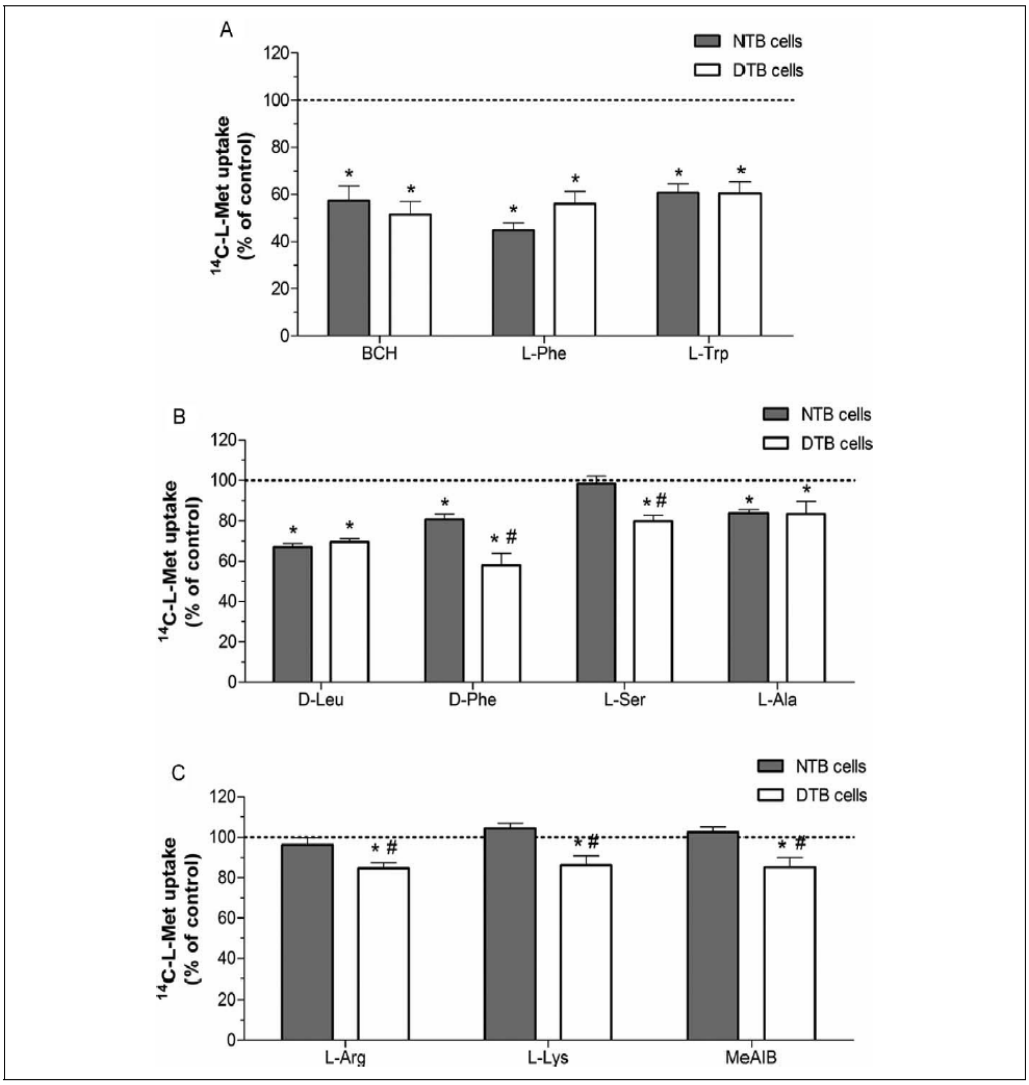

Figure 3. Pharmacological characterization of ^{14}C-L-methionine (^{14}C-L-Met) uptake in normal trophoblast (NTB) and diabetic trophoblast (DTB) cells. Initial rates of uptake were determined in cells incubated at 37°C with 250 nmol/L ^{14}C-L-Met for 6 minutes in the absence (control; corresponding to 100%) or in the presence of (A) 1 mmol/L 2-amino-2-norbornanecarboxylic acid (BCH), 100 μmol/L L-phenylalanine (L-Phe), or 100 μmol/L L-tryptophan (L-Trp), (B) 100 μmol/L D-leucine (D-Leu), 100 μmol/L D-phenylalanine (D-Phe), 100 μmol/L L-serine (L-Ser), or 100 μmol/L L-alanine (L-Ala), and (C) 100 μmol/L L-arginine (L-Arg), 100 μmol/L L-lysine (L-Lys), or 1 mmol/L α-(methylamino)isobutyric acid (MeAIB). Shown is arithmetic mean ± standard error of the mean (n = 5-9 from 2 to 3 distinct placenta). *Significantly different from control ($P < .05$) and #significantly different from uptake by NTB cells ($P < .05$).

uptake in NTB and DTB cells were also found. Namely, the inhibitory effect of D-Phe was more pronounced in DTB when compared to NTB cells (42% vs 19% inhibition, respectively; Figure 3B) and L-Ser, L-Arg, L-Lys, and MeAIB, which were devoid of the effect upon ^{14}C-L-Met uptake in NTB cells, were able to decrease ^{14}C-L-Met uptake (by 15%-20%) in DTB cells (Figure 3B and C).

As a whole, these results indicate that system L (represented by the Na^+-independent and BCH-, L-Phe-, and L-Trp-sensitive component) seems to play an important role in ^{14}C-L-Met

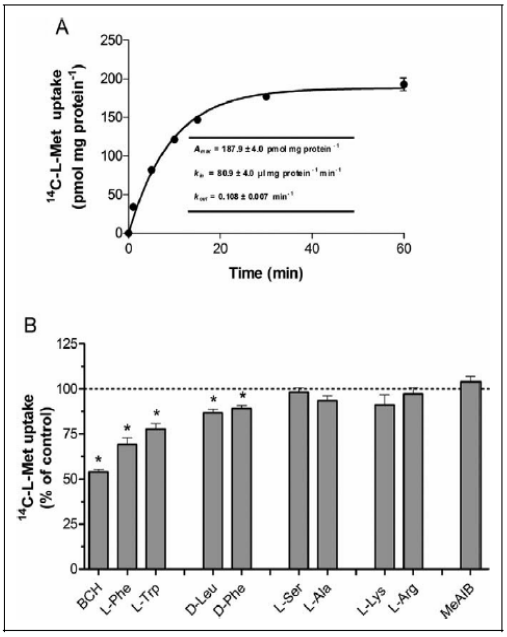

Figure 4. Time course (A) and characterization (B) of ^{14}C-L-methionine (^{14}C-L-Met) uptake by Bewo cells. For time course experiments, the cells were incubated for various periods of time at 37°C with 250 nmol/L ^{14}C-L-Met, at pH 7.5 (n = 8). Analysis of the time course allowed the determination of the steady state accumulation (A_{max}) and the rate constant for inward (k_{in}) and outward (k_{out}) transport. For the characterization experiments, initial rates of uptake were determined in cells incubated at 37°C with 250 nmol/L ^{14}C-L-Met for 6 minutes in the absence (control; corresponding to 100%) or in the presence of 1 mmol/L 2-amino-2-norbornanecarboxylic acid (BCH), 100 µmol/L L-Phe, 100 µmol/L L-Trp, 100 µmol/L D-Leu, 100 µmol/L D-Phe, 100 µmol/L L-Ser, 100 µmol/L L-Ala, 100 µmol/L L-Lys, 100 µmol/L L-Arg, or 1 mmol/L α-(methylamino)isobutyric acid (MeAIB; n = 6-12). Shown is arithmetic mean ± standard error of the mean. *Significantly different from control ($P < .05$).

uptake in both NTB and DTB cells, although some differences concerning the contribution of the 2 isoforms, LAT1 and LAT2, are apparent (D-Phe-sensitive LAT1 isoform seems to be more active in NTB cells, and L-Ser-sensitive LAT2 isoform seems to be functionally present only in DTB cells). The results also indicate that systems A (a Na^+-dependent and MeAIB-sensitive system), b^{0+} (a Na^+-independent and L-Lys- and L-Arg-sensitive system), and y^+L (a Na^+-dependent and BCH-insensitive component) may contribute to ^{14}C-L-Met uptake in DTB cells.

Quantification of mRNA Levels of Amino Acid Transporters in NTB and DTB Cells

In order to investigate whether the differences in the pharmacological characteristics of ^{14}C-L-Met uptake in NTB and DTB cells result from differences in the transcriptional level of amino acid transporters, we compared the steady state mRNA levels of some transporters in NTB and DTB cells, by qRT-PCR.

The genes encoding the following large neutral amino acid transporters, which seemed by our uptake results to be differentially active in NTB and DTB cells, were quantified: Na^+-coupled neutral amino acid transporter 1 (SNAT1) and SNAT2, L-type amino acid transporter 2 (LAT2), and y^+LAT1.

The mRNA expression levels of all the studied genes were not significantly different in NTB and DTB cells (the ratio test gene/β-actin was 61.2 ± 32.1 and 29.2 ± 9.8 for SNAT1, 26.2 ± 4.5 and 20.4 ± 4.7 for SNAT2, 24.7 ± 4.8 and 42.1 ± 12.8 for LAT2, and 0.25 ± 0.02 and 0.28 ± 0.04 for y^+LAT1, respectively; n = 6).

Characterization of ^{14}C-L-Met Uptake in Bewo Cells

In a second series of experiments, we characterized ^{14}C-L-Met uptake in Bewo cells in terms of time and Na^+ dependence and specificity of the carrier systems involved.

Time course and Na^+ dependence. As shown in Figure 4A, Bewo cells accumulated ^{14}C-L-Met in a time-dependent way, uptake being linear for the first 6 minutes of incubation. On the basis of this information, subsequent experiments were performed using a 6-minute incubation time. Analysis of the time course allowed determination of k_{in}, k_{out}, and A_{max} values, which are shown in Figure 4A.

Next, we verified that ^{14}C-L-Met transport in Bewo cells was only slightly Na^+ dependent, as substitution of Na^+ with either Li^+ or Ch^+ caused only a 6% to 7% decrease in uptake (results not shown).

Pharmacological characterization. The specificity of the carrier system involved in ^{14}C-L-Met uptake was also investigated in Bewo cells. Characterization revealed that ^{14}C-L-Met uptake was strongly (±50%) reduced by BCH, less markedly inhibited (by 20%-30%) by L-Phe and L-Trp, and only slightly (11%-13%) inhibited by D-Leu and D-Phe (Figure 4B). On the contrary, ^{14}C-L-Met uptake was not changed by any of the other amino acids tested (namely L-Ser, L-Ala, L-Lys, and L-Arg) nor by MeAIB (Figure 4B). As a whole, these results indicate that ^{14}C-L-Met uptake in Bewo cells is mainly system L-mediated.

Effect of GDM-Associated Conditions Upon ^{14}C-L-Met Uptake in Bewo Cells

Concentration and time dependence. In this set of experiments, we investigated the effect of exposure to distinct concentrations of some specific GDM-associated conditions, for different time periods, upon the uptake of ^{14}C-L-Met by Bewo cells.

As can be seen in Figure 5, exposure of the cells for 48 to 72 hours to 10 mmol/L D-glucose (corresponding to an hyperglycemic situation)[7] decreased ^{14}C-L-Met transport by a maximum of 15%.

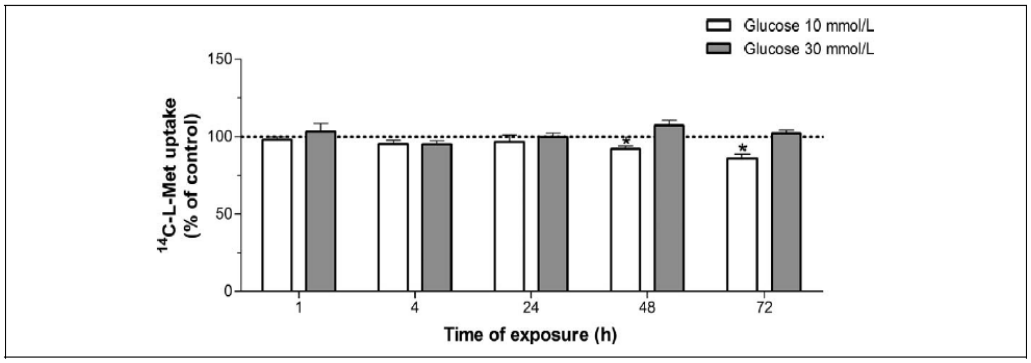

Figure 5. Effect of hyperglycemia upon ^{14}C-L-methionine (^{14}C-L-Met) uptake by Bewo cells. Cells were exposed to 10 or 30 mmol/L D-glucose (n = 6-13) or mannitol (control; corresponding to 100%) for 1 to 72 hours, and initial rates of uptake were then determined by incubating cells for 6 minutes at 37°C in buffer with 250 nmol/L ^{14}C-L-Met. Shown are arithmetic mean ± standard error of the mean (n = 6-13). *Significantly different from control ($P < .05$).

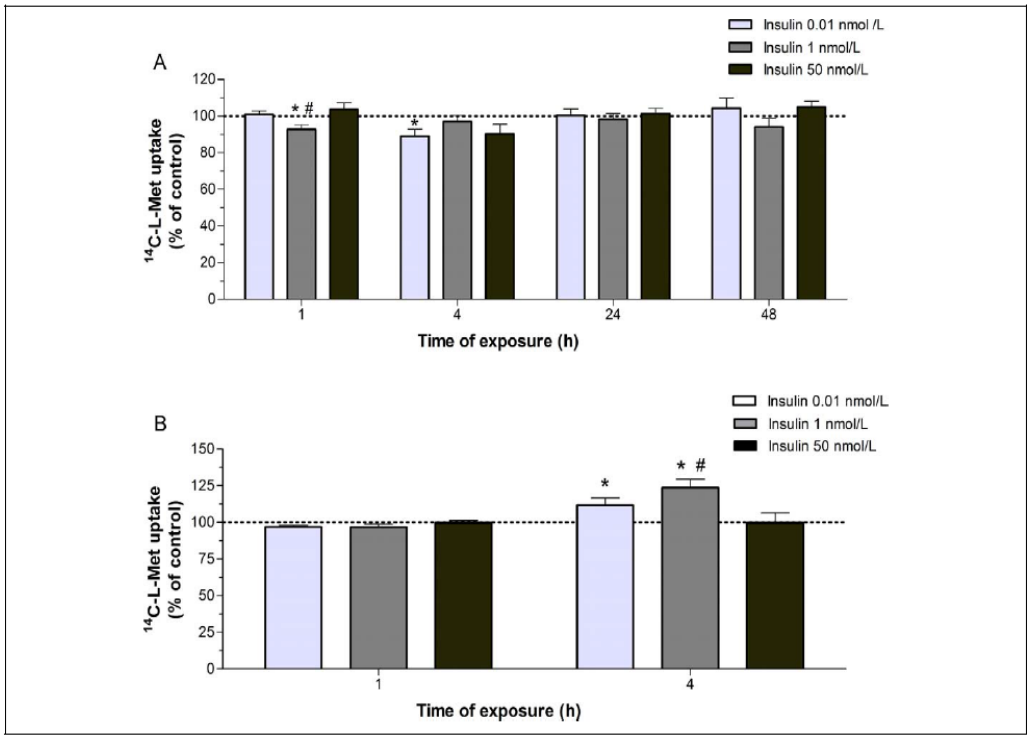

Figure 6. Effect of insulin upon ^{14}C-L-methionine (^{14}C-L-Met) uptake by Bewo cells. (A) Cells were exposed to 0.01, 1, or 50 nmol/L insulin or the respective solvent (control; corresponding to 100%) for 1 to 48 hours, and initial rates of uptake were then determined by incubating cells for 6 minutes at 37°C in buffer with 250 nmol/L ^{14}C-L-Met (n = 9-14); (B) cells were exposed to 0.01, 1, or 50 nmol/L insulin or the respective solvent (control, corresponding to 100%) for 1 or 4 hours, and initial rates of uptake were then determined by incubating cells for 6 minutes at 37°C in fetal calf serum (FCS)-free culture medium with 250 nmol/L ^{14}C-L-Met (n = 8-13). Shown is arithmetic mean ± standard error of the mean. *Significantly different from control ($P < .05$) and #significantly different from insulin 0.01 nmol/L ($P < .05$).

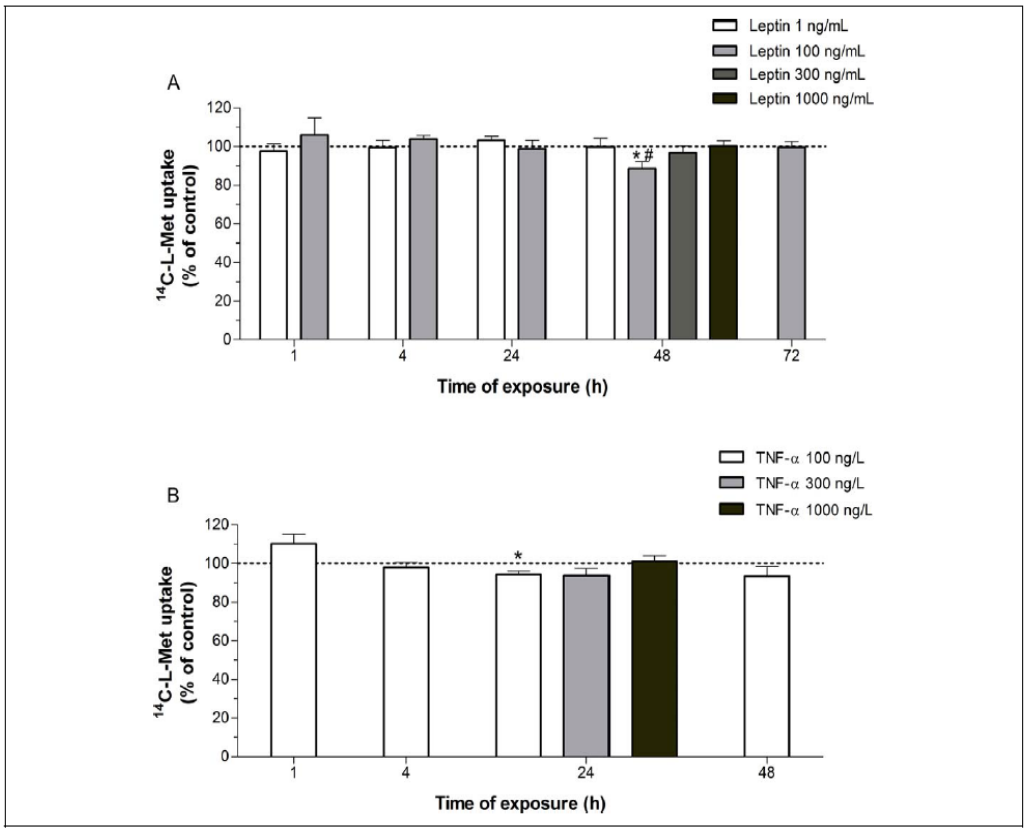

Figure 7. Effect of leptin and tumor necrosis factor-α (TNF-α) upon ^{14}C-L-methionine (^{14}C-L-Met) uptake by Bewo cells. (A) Cells were exposed to 1, 100, 300, or 1000 ng/mL leptin or the respective solvent (control; corresponding to 100%) for 1 to 72 hours, and initial rates of uptake were then determined by incubating cells for 6 minutes at 37°C with 250 nmol/L ^{14}C-L-Met (n = 5-13); (B) cells were exposed to 100, 300, or 1000 ng/L TNF-α or the respective solvent (control; corresponding to 100%) for 1 to 48 hours, and initial rates of uptake were then determined by incubating cells for 6 minutes at 37°C with 250 nmol/L ^{14}C-L-Met (n = 5-16). Shown is arithmetic mean ± standard error of the mean. *Significantly different from control ($P < .05$) and #significantly different from leptin (1 ng/mL; $P < .05$).

Exposure of the cells for short periods (1 and 4 hours) to 0.01 to 1 nmol/L insulin (normoinsulinemia conditions)[34] caused a modest (±10%) but significant decrease in ^{14}C-L-Met uptake (Figure 6A). Interestingly enough, when uptake of ^{14}C-L-Met was carried out in FCS-free culture media, instead of buffer as above, a 4-hour exposure to 0.01 to 1 nmol/L insulin increased ^{14}C-L-Met uptake (Figure 6B).

Exposure for 48 hours to 100 ng/mL leptin, a concentration known to be representative of hyperleptinemia in GDM,[2,26] caused a 12% decrease in ^{14}C-L-Met uptake in Bewo cells, when compared with control and normoleptinemic (1 ng/mL leptin) conditions[35] (Figure 7A).

In relation to the effect of proinflammatory mediators (LPS and TNF-α), TNF-α did not alter the uptake of ^{14}C-L-Met, with the exception of a small decrease (6%) observed after treatment of Bewo cells for 24 hours with 100 ng/L of this cytokine (Figure 7B), which is a concentration within the range found in GDM.[36] Finally, exposure of the cells to LPS (1 µg/mL) for 1 to 48 hours did not affect ^{14}C-L-Met uptake, as also did exposure of the cells for 1 hour to higher concentrations of this agent (10 and 50 µg/mL; results not shown). The range of concentrations of LPS tested are known to stimulate proinflammatory cytokines (interleukin 6 and TNF-α) secretion in TB cells.[37]

Characterization of the effects of hyperglycemia and hyperleptinemia. In this set of experiments, we further characterized the inhibitory effect of hyperglycemia (10 mmol/L; 72 hours) and hyperleptinemia (100 ng/mL; 48 hours) upon ^{14}C-L-Met uptake.

First, we examined their effect upon the kinetic parameters of ^{14}C-L-Met uptake. For this, uptake of ^{14}C-L-Met (0.25-50 µmol/L) was measured, either in the absence or in the presence of these conditions. We verified that both the K_m and V_{max}

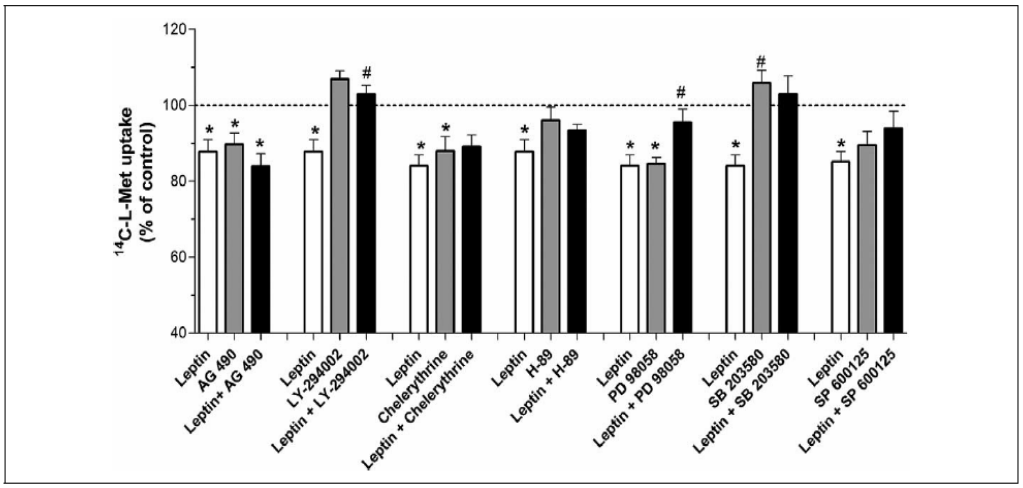

Figure 8. Effect of the inhibitors of intracellular signaling pathways upon hyperleptinemia-induced inhibition of ^{14}C-L-methionine (^{14}C-L-Met) uptake by Bewo cells. Initial rates of uptake were determined in cells incubated for 6 minutes with ^{14}C-L-Met 250 nmol/L, after treatment for 48 hours with leptin 100 ng/mL (leptin), AG 490 5 μmol/L (AG 490), leptin 100 ng/mL + AG 490 5 μmol/L (leptin + AG 490), LY-294002 1 μmol/L (LY-294002), leptin 100 ng/mL + LY-294002 1 μmol/L (leptin + LY-294002), chelerythrine 0.1 μmol/L (chelerythrine), leptin 100 ng/mL + chelerythrine 0.1 μmol/L (leptin + chelerythrine), H-89 1 μmol/L (H-89), leptin 100 ng/mL + H-89 1 μmol/L (leptin + H-89), PD 98058 2.5 μmol/L (PD 98058), leptin 100 ng/mL + PD 98058 2.5 μmol/L (leptin + PD 98058), SB 203580 9.6 μmol/L (SB 203580), leptin 100 ng/mL + SB 203580 9.6 μmol/L (leptin + SB 203580), SP 600125 5 μmol/L (SP 600125), and leptin 100 ng/mL + SP 600125 5 μmol/L (leptin + SP 600125; n = 9-13). Shown is arithmetic mean ± standard error of the mean. *Significantly different from the respective control ($P < .05$) and #significantly different from leptin (100 ng/mL; $P < .05$).

values of ^{14}C-L-Met uptake (179.5 ± 79.6 μmol/L and 41.3 ± 14.9 nmol mg/prot/6 min, respectively; n = 6) were not significantly changed by any of the treatments (results not shown).

Then, we analyzed the inhibitory effect of hyperglycemia and hyperleptinemia upon ^{14}C-L-Met uptake in the presence of the system L substrate BCH (1 mmol/L). Our results strongly suggest that these conditions reduced system L-mediated ^{14}C-L-Met uptake (results not shown; n = 8-9), since the inhibitory effect of BCH plus glucose or leptin was similar to the inhibitory effect of BCH alone.

In the final part of this work, we investigated the intracellular signaling mechanisms involved in the inhibitory effect of hyperglycemia and hyperleptinemia upon ^{14}C-L-Met uptake.

The role of mammalian target of rapamycin (mTOR) in hyperglycemia (10 mmol/L; 72 hours)-induced inhibition of ^{14}C-L-Met uptake was investigated using a specific mTOR inhibitor, rapamycin. No significant change was found in ^{14}C-L-Met uptake with rapamycin alone, and the effect of D-glucose was not changed in the presence of this compound (results not shown; n = 9).

The functions attributed to leptin depend upon its binding to OB-R leptin receptors, which have been localized in the human STB,[26] resulting in the activation of the following signal transduction pathways: phosphoinositide 3-kinase (PI3K), protein kinases A, B, and C, mitogen-activated protein kinases (MAPKs; extracellular-signal-regulated kinase 1/2 [ERK/MEK 1/2], Jun-NH_2-terminal kinase [JNK], and p38 MAPK), and janus kinases (JAKs)/signal transducers and activators of transcription (STAT; including JAK1 and 2 and STAT2, 3, and 5).[38]

The role of JAK2/STAT3 in hyperleptinemia (100 ng/mL; 48 hours)-induced inhibition of ^{14}C-L-Met uptake was investigated by treating Bewo cells for 48 hours with 5 μmol/L of JAK2 inhibitor AG 490. When tested alone, this agent was able to inhibit (by about 10%) ^{14}C-L-Met uptake, indicating that a certain basal JAK2/STAT3 activation is required for ^{14}C-L-Met transport activity. However, AG 490 was not able to reverse the inhibitory effect of hyperleptinemia upon ^{14}C-L-Met uptake (Figure 8).

We also investigated the effect of the PI3K inhibitor (LY-294002), of the specific PKA and PKC inhibitors H-89 and chelerythrine, respectively, and of specific inhibitors of ERK/MEK 1/2 (PD 98059), p38 MAPK (SB 203580), and JNK (SP600125), upon the inhibitory effect of hyperleptinemia (100 ng/mL; 48 hours) on ^{14}C-L-Met uptake.

As can be seen in Figure 8, ^{14}C-L-Met uptake was not affected by LY-294002, H-89, SB 203580, nor by SP600125, indicating that the activation of PI3K, PKA, p38 MAPK, and JNK is not necessary for ^{14}C-L-Met uptake by Bewo cells. On the contrary, uptake of ^{14}C-L-Met was reduced (by 12%-15%) in the presence of chelerythrine and PD 98059, indicating that a certain basal PKC and ERK/MEK 1/2 activation is required for the uptake of this amino acid.

Interestingly enough, the inhibitory effect of hyperleptinemia upon ^{14}C-L-Met uptake in Bewo cells was abolished by

LY-294002, PD 98059, and SB 203580, indicating that the inhibitory effect of hyperleptinemia depends on PI3K, ERK/MEK 1/2, and p38 MAPK, respectively (Figure 8).

Discussion

Changes in placental nutrient transport may have important consequences for the intrauterine programming of metabolic and cardiovascular diseases for several reasons: (1) changes in TB nutrient transporter activity constitute an important determinant of fetal growth and development[1]; (2) epigenetic regulation of fetal and placental genome, in particular gene methylation and histone modification, plays an important role in programming the fetus for future diseases[3]; and biological methylation reactions are dependent on the availability of amino acids, such as L-Met, and cofactors, which include folates, vitamin B_{12}, and choline[14]; and (3) placental nutrient transporters or their regulators may be by themselves key targets for epigenetic modification. Because changes in placental transport of methyl donors alter the availability of these compounds to the fetus, providing a direct link between placental function and structure, gene methylation and fetal programming,[3] we decided to investigate the possibility that, in GDM, changes in L-Met placental uptake are functionally present.

For this, we first determined the characteristics of ^{14}C-L-Met uptake in normal human TBs, using 2 cellular models of human TBs: TB cells and the Bewo cell line. Uptake of ^{14}C-L-Met by both NTB and Bewo cells shows similar characteristics: (1) it is time dependent, (2) it exhibits carrier-mediated kinetics, with a similar affinity (in the micromolar range) for ^{14}C-L-Met, (3) it is mainly Na^+ independent (although the Na^+-dependent component is greater in NTB [25%] than in Bewo cells [7%]), and (4) it is inhibited by BCH, L-Phe, L-Trp, D-Leu, and D-Phe but not by L-Ser, L-Arg, L-Lys, and MeAIB. However, in a distinct way, ^{14}C-L-Met uptake is inhibited by L-Ala in NTB cells only. Taken together, it is concluded that ^{14}C-L-Met uptake in NTB and Bewo cells is mainly system L mediated (represented by the Na^+-independent and BCH-, L-Phe-, and L-Trp-sensitive component) more specifically its D-Phe- and D-Leu-sensitive LAT1 isoform, although a small contribution of the Na^+-dependent, BCH-insensitive and L-Ala-sensitive transporter y^+L (y^+LAT2 isoform) is also present in NTB but not in Bewo cells. The lack of inhibition of ^{14}C-L-Met uptake in NTB cells by the system y^+L substrates L-Arg and L-Lys is probably due to the overlying activity of the cationic amino acid transport system y^+, which makes the major contribution to the placental uptake of these amino acids,[20] thereby reducing their efficacy to inhibit system y^+L-mediated ^{14}C-L-Met uptake.

Because ^{14}C-L-Met uptake in Bewo cells is almost totally Na^+ independent but was only half abolished by BCH, it can be speculated that another Na^+-independent and BCH-insensitive transporter might participate in ^{14}C-L-Met uptake. This may well correspond to LAT3[39] and/or LAT4[40] system L isoforms, both of which are expressed in the human placenta[41] and are sensitive to BCH in concentrations higher than 1 mmol/L,[39,40] and/or to system y^+, a Na^+-independent, and BCH-insensitive transporter present in many tissues including the human placenta,[5] which was recently proposed to participate in L-Met uptake in Caco-2 cells.[42]

Based on the functional characteristics of amino acid transporters, such as substrate specificity, inhibition by L-Met and placental presence, placental L-Met uptake would be hypothesized to occur by systems L, b^{0+}, y^+L,[5,6,20,21] and system A.[20,21] Interestingly, our results show that LAT1 and y^+LAT2 seem to be functionally the most important L-Met transporters at the placental level in a normal situation.

We also investigated the influence of GDM and specific GDM-associated conditions upon ^{14}C-L-Met uptake. For this, 2 distinct approaches were used, a comparison between ^{14}C-L-Met uptake in TB cells obtained from normal and GDM pregnancies (NTB and DTB cells, respectively) and an investigation of the effects of high glucose, insulin, leptin, and proinflammatory mediators (LPS and TNF-α) in Bewo cells.

Comparison between ^{14}C-L-Met uptake in NTB and DTB cells shows a similar profile of time dependence, kinetics, Na^+ independence (75% in both cell types), and sensitivity to potential inhibitors. In contrast to our results, an in vivo study demonstrated that fetal umbilical plasma concentrations of L-Met is higher in GDM compared with normal pregnancies, in the absence of significant differences in maternal plasma concentrations.[43] However, placental transfer of L-Met was not measured in that study and an alteration in placental metabolism or fetal metabolism and/or excretion of L-Met cannot be ruled out.

Based on the comparison of ^{14}C-L-Met uptake in NTB and DTB cells, we conclude that system L (represented by the Na^+-independent and BCH-, L-Phe-, and L-Trp-sensitive component), and more specifically its D-Phe- and D-Leu sensitive LAT1 isoform, seems to play an important role in ^{14}C-L-Met uptake in both NTB and DTB cells. However, a contribution of system A (a Na^+-dependent and MeAIB-sensitive system), LAT2 (a L-Ser-sensitive system L isoform), and system b^{0+} (a Na^+-independent and L-Lys- and L-Arg-sensitive system), and a higher contribution of the Na^+-dependent and BCH-insensitive system y^+L (possibly y^+LAT1, because of the similar inhibitory effect of the y^+LAT2 substrate L-Ala in both NTB and DTB cells) appears to exist in DTB cells only. Although L-Met is not a preferential system A substrate, other authors have also demonstrated that system A may transport L-Met.[44] Interestingly, our results suggest that L-Met becomes a system A substrate in GDM. Analysis of mRNA levels of SNAT1 and SNAT2 (the major system A transporters present in STB during late gestation, with higher affinity for L-Met as compared to SNAT4[45]), LAT2 and y^+LAT1 showed no significant differences between NTB and DTB cells. We thus conclude that these transporters are not transcribed at different levels in NTB and DTB cells; rather, posttranscriptional changes at the protein level or changes in the intrinsic activity of the transporters probably occur.

Data available on placental transport of amino acids in GDM pregnancies are conflicting: an increase in systems A and L activity but not in the transport of the essential amino acids lysine and taurine,[19] a decrease[18] or no alteration in system

A activity,[16] and no alteration in system L activity[17] has been described. The contrasting findings between these studies may be the result of differences in study populations (different mother's age, ethnicity, GDM diagnostic criteria, metabolic control, therapeutics of GDM, incidence of large-for-gestational-age infants/fetal macrosomia, and different fetal and placental weights). Fetal macrosomia, a very common complication of GDM, is associated with an alteration in the placental transfer of amino acids.[18,19] It would be interesting to compare L-Met transport in DTB cells isolated from GDM pregnancies with adequate for gestational age and macrosomic babies. However, the small number of GDM placenta with macrosomic babies present in our GDM group did not allow us to perform that comparison.

Moreover, compared with these previous reports, our results concerning system L activity agree with those of Nandakumaran et al,[17] whereas those concerning system A activity agree with those of Jansson et al.[19]

Hyperglycemia, hyperinsulinemia,[7] hyperleptinemia,[8] and increased inflammation[9] are commonly found in GDM. Moreover, the placental gene expression of inflammatory response-associated genes, including TNF-α and leptin, are significantly upregulated in GDM.[46] So, in the last part of this work, an investigation of the effects of short- and long-term exposure to high glucose, insulin, leptin, and proinflammatory mediators (LPS and TNF-α) in Bewo cells was made. Bewo cells, a known cellular model of the human STB,[23] have nutrient transport mechanisms very similar to NTB cells[47] and have been used to investigate the placental transport of neutral amino acids, because they express the same polarized amino acid transport systems as normal human TBs.[48] As shown in this work, uptake of ^{14}C-L-Met by NTB and Bewo cells has very similar characteristics, and for these particular studies, Bewo cells have clear advantages over NTB cells due to their greater stability, life span, viability with passage, easier maintenance,[49] and absence of patient variability.

Short-term (1-4 hours) exposure of Bewo cells to 0.01 and 1 nmol/L insulin (normoinsulinemic conditions)[34] caused a modest (±10%) decrease in ^{14}C-L-Met uptake, but a higher concentration of insulin (50 nmol/L), corresponding to supraphysiological levels,[2,26] did not affect the uptake. Moreover, long-term exposure of these cells to normo- and hyperinsulinemic conditions did not affect ^{14}C-L-Met uptake. Because ^{14}C-L-Met uptake in Bewo cells is mainly system L mediated, we conclude that both short- and long-term hyperinsulinemic conditions do not affect system L-mediated placental amino acid uptake.

When uptake of ^{14}C-L-Met was carried out in FCS-free culture media, a 4-hour exposure to 0.01 and 1 nmol/L insulin increased ^{14}C-L-Met uptake by 10% to 20%. In comparison with incubation buffer, FCS-free culture medium contains a mixture of amino acids, including L-Met, in very high concentrations (0.02-2.4 mmol/L each). It is thus probable that this condition may interfere with the effect of insulin upon ^{14}C-L-Met uptake.

Long-term (24 hours) exposure of Bewo cells to TNF-α (100 ng/L) caused a very small (6%) decrease in uptake of ^{14}C-L-Met, leading us to conclude that this proinflammatory cytokine does not seem to have a great impact upon system L-mediated placental amino acid transport. In agreement with these results, it was reported that physiological concentrations of TNF-α stimulate the activity of system A but not of system L, in NTB cells.[50] In contrast, in nonplacental cell types, TNF-α is known to affect the transport of neutral[51] amino acids. Similarly, LPS, used in concentrations known to induce increased cytokines (including TNF-α) secretion by TB cells,[37] was also devoid of any effect on ^{14}C-L-Met uptake. To the best of our knowledge, this is the first report about the effect of LPS upon amino acid uptake in human TB cells. However, in cell types of nonplacental origin, LPS was found to alter the uptake of amino acids.[52]

Long-term (48-72 hours) exposure of Bewo cells to 10 mmol/L glucose (corresponding to an hyperglycemic situation)[7] caused an 8% to 15% decrease in ^{14}C-L-Met uptake, and long-term (48 hours) exposure to 100 ng/mL leptin (corresponding to an hyperleptinemic situation) caused a 12% decrease in ^{14}C-L-Met uptake.

Further experiments aimed at elucidating the mechanisms involved in the effect of glucose (10 mmol/L; 72 hours) and leptin (100 ng/mL; 48 hours) upon ^{14}C-L-Met uptake revealed that both of these conditions appear to inhibit system L-mediated ^{14}C-L-Met uptake, although none of them affected the kinetics of uptake. This last observation suggests that transcriptional or translational mechanisms may constitute a probable explanation for their effect. This hypothesis can also explain the different short- and long-term effects observed with these GDM-associated conditions upon ^{14}C-L-Met uptake, because these conditions may elicit long-term adaptative changes in system L mRNA or protein expression levels, which may not be induced by short-term exposure.

The mTOR is a serine/threonine kinase involved in cell growth and metabolism, whose actions are regulated by a multitude of intracellular and extracellular signals, including hormones, growth factors, and nutrients.[53] The inhibitory effect of glucose upon ^{14}C-L-Met uptake was found to be mTOR independent. This conclusion stands in contrast to a recent publication, in which glucose regulation of systems A and L and taurine transporters in NTB cells were mTOR dependent.[53] This discrepancy is probably related to the fact that different cell models were used,: syncytial primary cultures of human cytotrophoblasts in the work of Roos et al[53] and Bewo cells not pretreated with cyclic adenosine monophospahate stimulators, to induce syncytialization, in the present work. Because TB differentiation/syncytialization is accompanied by changes in the activity, expression, and polarization of neutral amino acid transporters (including system L),[48] the difference in the syncycial status of the 2 cell models may explain this discrepancy.

Finally, the inhibitory effect of leptin upon ^{14}C-L-Met uptake was found to be dependent on PI3K and MAP kinases ERK/MEK 1/2 and p38 MAPK. This conclusion is in good agreement with the fact that these signal transduction pathways, which are important signal transduction pathways activated by

leptin,[38] are known to regulate the activity of amino acid transporters in Bewo cells.[54] Studies available concerning leptin regulation of placental amino acid transport focused in system A and state that its activity is upregulated by leptin in human placental villous fragments, via activation of JAK2-STAT3, at concentrations higher than 100 ng/mL.[2,26]

In summary, we show that ^{14}C-L-Met uptake in NTB and Bewo cells is mainly system L (LAT1) mediated, although a small contribution of the Na^+-dependent, L-Ala-sensitive y^+L system (most probably its y^+LAT2 isoform) is functionally present in NTB cells. Comparison of ^{14}C-L-Met in NTB and DTB cells shows similar kinetics of ^{14}C-L-Met uptake, but in DTB cells, a contribution of system A, LAT2, and possibly system b^{0+}, and a higher contribution of system y^+L (probably the y^+LAT1 isoform) appears to exist in relation to NTB cells. However, we did not find significant changes in the steady state mRNA levels of system A (SNAT1 and SNAT2 isoforms), LAT2, and y^+LAT1. Given the broad substrate specificities of system A (small neutral amino acids), systems b^{0+} and y^+L (cationic and neutral amino acids), and system L (large neutral amino acids), our results suggest that the placental uptake of other neutral amino acids and even of cationic amino acids can be altered in GDM pregnancies, thereby changing placental and fetal amino acid delivery. Interestingly enough, the tendency for the higher placenta and newborn weight in the GDM group may well be associated with such an alteration in amino acid delivery. So, more research is needed in order to identify potential changes in amino acid transport across the GDM placenta. Finally, short-term exposure of Bewo cells to insulin (0.01-1 nmol/L) and long-term exposure to high glucose (10 mmol/L), TNF-α (100 ng/L), or leptin (100 ng/mL) decreased ^{14}C-L-Met uptake by 6% to 15%. High-glucose (10 mmol/L; 72 hours) and leptin (100 ng/mL; 48 hours) appear to inhibit system L-mediated uptake of ^{14}C-L-Met but did not change the kinetics of uptake of the amino acid. The effect of high glucose was found to be mTOR independent, and the effect of leptin was found to be PI3K, ERK/MEK 1/2, and p38 MAPK dependent. Because our results suggest that uptake of L-Met in Bewo cells is mainly system L mediated, we can hypothesize that uptake of other neutral amino acids can also be potentially affected by specific GDM conditions.

A last point to discuss is the apparently contrasting observation that uptake of ^{14}C-L-Met is quantitatively similar in NTB and DTB cells, but that some of the GDM-associated conditions affect the uptake of this amino acid by Bewo cells. This suggests that the effect of GDM upon L-Met uptake cannot be mimicked by an isolated GDM-associated metabolic condition, being rather the result of simultaneous and interacting distinct factors.

In conclusion, uptake of ^{14}C-L-Met in NTB and DTB cells, although quantitatively similar, may involve the interplay of different transporters. Moreover, GDM-associated conditions cause a small, but significant decrease in ^{14}C-L-Met uptake in Bewo cells. As a whole, these results suggest that GDM, a common pregnancy disease, could have consequences in terms of amino acid delivery to the fetal–placental unit.

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Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Fundação para a Ciência e a Tecnologia (FCT) and COMPETE, QREN and FEDER (PTDC/SAU-OSM/102239/2008, SFRH/BD/63086/2009, and SFRH/BPD/40170/2007).

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Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Fundação para a Ciência e a Tecnologia (FCT) and COMPETE, QREN and FEDER (PTDC/SAU-OSM/102239/2008, SFRH/BD/63086/2009, and SFRH/BPD/40170/2007).

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Journal of Nutritional Biochemistry 24 (2013) 1741–1750

Journal of Nutritional Biochemistry

Gestational diabetes mellitus decreases placental uptake of long-chain polyunsaturated fatty acids: involvement of long-chain acyl-CoA synthetase☆

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Received 3 September 2012; received in revised form 31 January 2013; accepted 7 March 2013

Abstract

The long-chain polyunsaturated fatty acids (LC-PUFAs) arachidonic (AA) and docosahexaenoic (DHA) acids are essential for fetal development. Gestational diabetes mellitus (GDM) is a pregnancy disorder associated with perinatal and lifelong risk complications for both the mother and the newborn. Our aim was to investigate the influence of GDM, and some of its associated conditions, upon the placental uptake of AA and DHA. Uptake of ^{14}C-AA and ^{14}C-DHA by human trophoblasts obtained from normal pregnancies (NTB cells) was mediated by both saturable (for lower substrate concentrations) and non-saturable (for higher substrate concentrations) mechanisms. Uptake of both fatty acids was inhibited by other LC-PUFAs and, markedly, by the long-chain acyl-CoA synthetase (ACSL) inhibitor, triacsin C. Human trophoblasts obtained from GDM pregnancies (DTB cells) showed a significantly lower ^{14}C-AA and ^{14}C-DHA accumulation, through a decrease in both the saturable and the non-saturable components of uptake, which was associated with a decrease in ACSL1 mRNA levels. Uptake of LC-PUFAs by NTB cells increased (by 20–25%) after short-term exposure to TNF-α (^{14}C-AA and ^{14}C-DHA) and insulin (^{14}C-DHA). In conclusion, GDM, distinctly from its associated conditions, markedly decreases placental uptake of LC-PUFAs, which probably contributes to the deleterious effects of this disease for the newborn.

Keywords: Gestational diabetes; Placentae; Transport; Long-chain polyunsaturated fatty acids

1. Introduction

The essential fatty acids linoleic (LA; 18:2n-6) and α-linolenic (18:3n-3) acids and their respective long-chain polyunsaturated fatty acids (LC-PUFAs) derivatives, arachidonic (AA; 20:4n-6) and docosahexaenoic acids (DHA; 22:6n-3), play a fundamental role during pregnancy for both the health of the pregnant woman and for the growth and development of the fetus [1].

Apart from serving as an energy substrate, LC-PUFAs are essential components of membrane lipids that maintain the structure and function of cellular and subcellular membranes and act as regulators of gene expression via nuclear receptors [2]. AA is essential for the synthesis of eicosanoids such as prostaglandins, thromboxanes and leukotrienes, which are important for the development of fetal nervous, visual, immune and vascular systems [3,4]. DHA is also essential for the development of the fetal neurovisual system, being incorporated in high concentrations in the brain and retina [3,4]. A deficiency of AA and DHA during intrauterine life is associated with brain abnormalities in neonates, and some evidence suggests that supplementation during pregnancy with these fatty acids may influence the visual, behavioral and cognitive functions of the offspring [5]. Moreover, increased consumption of LC-PUFAs during pregnancy is suggested to be beneficial for overall fetal development and to lower the risk of pre-term delivery and preeclampsia [6]. Based on this, a recommendation for DHA supplementation during pregnancy was made [7]. In humans, the vulnerable period of brain and retina development and the maximal rate of LC-PUFAs accumulation occurs during the third trimester of pregnancy and first 6–9 months of postnatal life [1], leading to the concept that the last trimester fetus and newborn infant are particularly vulnerable to irrevocable developmental deficits if AA and DHA supply is inadequate [4].

Since human fetuses synthesize insufficient amounts of LC-PUFAs, a considerable supply of these fatty acids must be obtained from the maternal circulation through the placentae, by transfer across the syncytiotrophoblast (STB) transporting epithelium [4,8,9]. Interestingly, LC-PUFAs are present at higher levels in fetal circulation, in relation to maternal circulation. This is thought to be the result of an active and selective placental transfer of these fatty acids in favor of the fetus [9]. Accordingly, there is a preferential and more efficient uptake of LC-PUFAs [DHA>AA>LA>α-linolenic acid>oleic acid (OA)] over other non-essential shorter fatty acids by human placental membranes and trophoblasts cell lines (Bewo cells) [10–12].

Molecular mechanisms of how placental LC-PUFAs transport occurs are complex and not fully known [4,13]. Although fatty acids

☆ This study was supported by Fundação para a Ciência e a Tecnologia (FCT) and COMPETE, QREN and FEDER (PTDC/SAU-OSM/102239/2008, SFRH/BD/63086/2009 and SFRH/BPD/40170/2007).

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http://dx.doi.org/10.1016/j.jnutbio.2013.03.003

are taken up into cells via passive diffusion, emerging reports indicate that certain plasma membrane-located fatty acid transporters are also implicated in this process. Additionally, intracellular fatty acid binding proteins are responsible for directing different fatty acids to their specific intracellular locations. At the STB epithelium, several fatty acid transport proteins may be involved in the uptake of maternal plasma LC-PUFAs: placental plasma membrane fatty acid binding protein (pFABPpm), fatty acid transport proteins (FATPs) and fatty acid translocase (FAT/CD36), but the precise way in which these membrane-associated proteins facilitate transmembrane passage of fatty acids is still a matter of speculation [4,8,9]. In addition, intracellular long-chain acyl-CoA synthetases (ACSLs), a group of cytosolic enzymes that prevent the efflux of the incorporated fatty acids by converting them into acyl-CoA derivatives for further esterification or β-oxidation [14], also appear to be involved in fatty acid uptake [4,8,9].

Gestational diabetes mellitus (GDM), defined as a degree of glucose intolerance with onset or first recognition during pregnancy, affects about 7% of all pregnancies [15]. Hallmarks of this disease, and also of type 2 diabetes, include hyperglycaemia, hyperinsulinaemia and insulin resistance [15]. Additionally, hyperleptinemia [16] and low-grade inflammation, due to elevated levels of pro-inflammatory cytokines [17], are also associated with these closely related diseases. Despite advances in treatment, GDM is associated with an unfavorable intrauterine environment to the fetus, increasing the risk of fetal macrosomia, the most common perinatal complication associated with this disease and a risk factor for operative delivery and traumatic birth injury. Furthermore, GDM presents programming effects, since it increases the risk for both the offspring and the mother to develop cardiovascular and metabolic diseases (obesity, type 2 diabetes mellitus and hypertension) later in life [18,19].

GDM and its metabolic conditions are known to be associated with specific changes in placental nutrient transport [17]. In relation to LC-PUFAs, neonates born from women with GDM or type 2 diabetes [20,21] have reduced plasma levels of AA and DHA. However, knowledge on the placental transport of fatty acids, and particularly LC-PUFAs, in GDM pregnancies remains scarcely studied, and not much is also known concerning the effect of GDM-associated conditions upon the placental uptake of LC-PUFAs.

Considering the important role played by LC-PUFAs in fetal visual, behavioral and cognitive development and that GDM appears to have programming effects, we hypothesize that this disease may interfere with the placental transport of LC-PUFAs. For this purpose, we decided to characterize AA and DHA uptake by primary cultured human cytotrophoblasts obtained from normal pregnancies (NTB cells), to compare it with uptake by primary cultured human cytotrophoblasts obtained from GDM pregnancies (DTB cells) and to test the effect of specific GDM-associated conditions [increased levels of glucose, insulin, leptin and tumor necrosis factor-α (TNF-α)] upon the uptake of these LC-PUFAs by NTB cells. Human primary cultured cytotrophoblasts are a suitable model to study placental transport because they spontaneously differentiate into a functional and polarized STB-like structure that retain all the cellular machinery of the in vivo STB [22].

2. Materials and methods

2.1. Materials

^{14}C-AA (arachidonic acid, [1-^{14}C]; specific activity 55 mCi/mmol) and ^{14}C-DHA (docosahexaenoic acid, 4,7,10,13,16,19-[1-^{14}C]; specific activity 55 mCi/mmol) (American Radiolabeled Chemicals, St. Louis, MO, USA), DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt), 2,4-dinitrophenol, phloretin, arachidonic acid (from porcine liver), *cis*-4,7,10,13,16,19-docosahexaenoic acid, *cis*-5,8,11,14,17-eicosapentaenoic acid, linoleic acid (LA), γ-linolenic acid (γ-LNA), OA, sodium palmitate, human insulin (recombinant, expressed in yeast), human TNF-α (recombinant, expressed in HEK293 cells), DMEM/F-12 culture medium, FCS (fetal calf serum), HEPES (*N*-2-hydroxyethylpiperazine–*N*′-2-ethanesulfonic acid), MES (2-[*N*-morpholino]ethanesulfonic acid) hydrate, bovine serum albumin (BSA), BSA essentially fatty acid free, DNase I (deoxyribonuclease I from bovine pancreas), Percoll and antibiotic/antimycotic solution (100 U/ml penicillin; 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B), NADH (β-nicotinamide adenine dinucleotide, reduced form), sodium pyruvate, pNPP (*p*-nitrophenylphosphate), pNP (*p*-nitrophenol), paraformaldehyde, sodium citrate tribasic dihydrate and TWEEN 20 (Sigma, St. Louis, MO, USA); D(+)-glucose, DMSO (dimethylsulfoxide), Triton X-100 and Tris [*tris*-(hydroxymethyl)-aminomethane hydrochloride] (Merck, Darmstadt, Germany); HBSS (Hank's balanced salt solution) and trypsin 2.5% (10× solution) (Gibco, Invitrogen, Carlsbad, CA, USA); recombinant human leptin (Invitrogen); D-mannitol (Difco Laboratories, Detroit, MI, USA); triacsin C (Alomone Labs, Jerusalem, Israel); Tripure isolation reagent and DAPI (4′,6-diamidino-2-phenylindole) (Roche Diagnostics, Mannheim, Germany).

The drugs to be tested were dissolved in water, 100% (v/v) ethanol, 50% (v/v) ethanol, DMSO, 0.01 mol/L HCl, 0.1% (w/v) BSA in water or 0.1% (w/v) BSA in phosphate-buffered saline (PBS). The final concentration of these solvents in the buffer and culture medium was 1% (v/v). Controls for the drugs were run in the presence of the solvent. Neither of the solvents had a significant effect on ^{14}C-AA and ^{14}C-DHA uptake (results not shown).

2.2. Collection of human placentae

Collection and processing of human placentae were approved by the Ethical Committee of Centro Hospitalar S. João, Porto. Human placentae were obtained at the Department of Obstetrics and Gynecology of Centro Hospitalar S. João, Porto, from uncomplicated (control, $n=10$) and GDM ($n=6$) term pregnancies (38–41 weeks), within half an hour after spontaneous delivery or elective caesarean section. Control placentae represented normal pregnancies with no associated maternal or fetal pathology and were collected at random.

In pregnant women without prior known diabetes, the diagnosis of GDM was performed by a two-step approach. All pregnant women were tested by a 50 g glucose challenge test at 24–28 weeks of gestation. In those with a blood glucose level ≥140 mg/dl (7.8 mmol/L) 1 h after the oral glucose load, a diagnostic oral glucose tolerance test (OGTT) was performed. GDM was diagnosed when two or more of the following plasma glucose concentrations were met or exceeded (according to the criteria of Carpenter and Coustan [23]): fasting blood glucose level ≥95 mg/dl (5.3 mmol/L) and/or blood glucose level ≥180 mg/dl (10 mmol/L), 155 mg/dl (8.6 mmol/L) or 140 mg/dl (7.8 mmol/L) 1, 2 or 3 h after a 100 g OGTT, respectively. These pregnancies were not associated with any major maternal or fetal pathology in addition to GDM. Women with diagnosed GDM were surveilled in Centro Hospitalar S. João and treated with diet and exercise therapy during the course of pregnancy up to the time of delivery. In three patients, insulin therapy was necessary. The criterion for initiating insulin therapy was the presence of a fasting blood glucose level ≥90 mg/dl (5 mmol/L) or a 2 h postprandial blood glucose level ≥120 mg/dl (6.7 mmol/L), despite consistent dietary and exercise adjustments. Selected clinical, anthropometric and demographic data for control or GDM study groups are given in Table 1 [24].

2.3. Primary cultures of human cytotrophoblasts (TB cells)

Villous cytotrophoblasts (TB cells) corresponding to control or GDM pregnancies (NTB or DTB cells, respectively) were isolated using a modification of the technique described by Kliman et al. [25], as previously described by our group [26,27]. After 3 days in culture, TB cells aggregate to form syncytial clumps corresponding to STB. This was confirmed by observing cell morphology with an optic microscope. To evaluate the purity of the TB cell cultures, cells in chamber slides were fixed with 4% (w/v) paraformaldehyde and immunolabeled with anti-vimentin and anti-cytokeratin antibodies. Ninety-five percent of the cells were cytokeratin positive, thus corresponding to epithelial TB cells.

2.4. Uptake of ^{14}C-arachidonic acid (^{14}C-AA) and ^{14}C-docosahexaenoic acid (^{14}C-DHA) by TB cells

For uptake studies, TB cells were seeded on 24-well plastic cell culture clusters at a cell seeding density of 14×10^5 cells/well, and the experiments were performed 3 days after the initial seeding. The experiments were performed in buffer with the following composition (in mmol/L): 125 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 12.5 HEPES–NaOH, 12.5 MES, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 5.6 mmol/L D(+)-glucose and 1% (w/v) essentially fatty acid-free BSA, pH 7.5 (unless otherwise stated). Initially, the culture medium was aspirated and the cells were washed with 0.3 ml buffer at 37°C; then the cell monolayers were preincubated for 20 min with 0.3 ml buffer at 37°C. Uptake was initiated by the addition of 0.2 ml buffer at 37°C containing 100 or 500 nmol/L ^{14}C-AA or ^{14}C-DHA (except in the experiments for determination of the kinetics of ^{14}C-AA and ^{14}C-DHA uptake, as indicated). Incubation was stopped after 6 min (unless otherwise stated) by removing the incubation buffer, placing the cells on ice and rinsing them with 0.5 ml ice-cold buffer without fatty acid-free BSA. The cells were then solubilized with 0.3 ml of 0.1% (v/v) Triton X-100 (in 5 mmol/L Tris–HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Table 1
Clinical, anthropometrical and demographic data of control and GDM study groups

	Control	GDM
Mothers		
n	10	6
Maternal age (years)	32.3±1.7	32.5±1.5
BMI[a] before delivery (kg/m^2)	26.6±1.5	32.8±1.3*
Gravida (*n*)	2.3±0.4	2.3±0.4
Parity (*n*)	0.9±0.3	1.2±0.4
Mode of delivery		
Vaginal [*n* (%)]	5 (50)	3 (50)
Cesarean [*n* (%)]	5 (50)	3 (50)
Therapeutics of GDM [*n* (%)]	–	Nutritional: 3 (50) Insulin: 3 (50)
Fasting blood glucose (mmol/L)	3.9±0.1[b]	4.6±0.3[b,*]
HbA_{1C} (%)[c]	–	5.5±0.2
Periconceptional folic acid use [*n* (%)][d]	9 (90)[e]	4 (67)[f]
Smokers [*n* (%)]	0 (0)[f]	0 (0)
Infants		
Gestational age at birth (weeks)[g]	39.6±0.3	39.3±0.3
Birth weight (g)[h]	3268.6±135.9	3382.5±196.1
Length[i] (cm)	48.5±0.5	49.7±0.6
SGA[j] newborn [*n* (%)]	1 (10)	0 (0)
AGA newborn [*n* (%)]	8 (80)	5 (83)
LGA newborn [*n* (%)]	1 (10)	1 (10)
Placental weight (g)	602.9±55.1	668.3±66.5
Gender [*n* (%)]	Male: 2 (20) Female: 8 (80)	Male: 2 (33) Female: 4 (67)
5-min Apgar score	9.4±0.2	9.2±0.3

Values represent means±S.E.M.
* Significantly different from control ($P<.05$).
[a] Parameter unknown for one subject.
[b] Values obtained at 24–28 weeks of gestation.
[c] Values obtained at 35–36 weeks of gestation. Parameter unknown for subjects from control group.
[d] Dosage and initiation period unknown.
[e] Parameter unknown for one subject.
[f] Parameter unknown for two subjects.
[g] Number of completed weeks at the time of delivery, determined by prenatal ultrasound at 11–13 weeks.
[h] Evaluated to the nearest gram.
[i] Evaluated to the nearest tenth of a centimeter.
[j] SGA, small for gestational age; AGA, adequate for gestational age; LGA, large for gestational age, according to published reference standards [23].

2.4.1. Pharmacological characterization of ^{14}C-AA and ^{14}C-DHA uptake by NTB and DTB cells

Compounds to be tested were present during both the preincubation (20 min) and incubation (6 min) periods.

2.4.2. Effect of GDM-associated conditions upon ^{14}C-AA and ^{14}C-DHA uptake by NTB cells

The effect of GDM-associated conditions upon ^{14}C-AA and ^{14}C-DHA uptake by NTB cells was tested by preincubating cells for 20 min (in culture media without FCS) and then incubating them for 6 min (in buffer) in the presence of D-glucose (20 mmol/L), insulin (50 nmol/L), leptin (100 ng/ml), TNF-α (100 ng/L) or the respective solvents. In 20 mmol/L glucose experiments, an isosmotic control was run using mannitol.

2.5. RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 3-day-old NTB and DTB cells (both seeded at a cell density of 10^7 cells/21 cm^2) using the Tripure isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Before cDNA synthesis, total RNA was treated with DNase I (Ambion Inc, Texas, USA) and 2 μg of the resulting DNA-free RNA was reverse transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen) in 80 μl of final reaction volume, according to manufacturer's instructions. Resulting cDNA was treated with RNase H (Invitrogen) to degrade unreacted RNA. For the qRT-PCR, 2 μl of the 80 μl reverse transcription reaction mixture was used.

For the calibration curve, placental standard cDNA was diluted in five different concentrations. qRT-PCR was carried out using a LightCycler (Roche, Nutley, NJ, USA). Twenty-microliter reactions were set up in microcapillary tubes using 0.5 μmol/L of each primer and 4 μl of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche, Mannheim, Germany). Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification [95°C for 10 s, annealing temperature (AT) for 10 s and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment] repeated 55 times, a melting curve program [(AT+10)°C for 15 s and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement] and a cooling step to 40°C for 30 s. ATs and sequence of primers are indicated in Table 2. The primer pair for β-actin was kindly donated by Dr. Joana Marques (Department of Genetics, Faculty of Medicine, University of Porto, Portugal). Data were analyzed using LightCycler 4.05 analysis software (Roche, Mannheim, Germany). The amount of mRNA of each studied gene was normalized to the amount of mRNA of the housekeeping gene (β-actin). There was no effect of GDM on the expression levels of β-actin (results not shown).

2.6. Quantification of cell viability [lactate dehydrogenase (LDH) activity assay]

NTB and DTB cells were seeded in 24-well plastic cell culture clusters (2 cm^2; diameter 16 mm; TPP) at a cell seeding density of 14×10^5 cells/well. After 3 days in culture, cell viability was determined by measuring the extracellular activity of the cytosolic enzyme LDH.

Cellular leakage of LDH into the extracellular culture medium was determined spectrophotometrically by measuring the decrease in absorbance of NADH during the reduction of pyruvate to lactate. LDH activity was expressed as the percentage of extracellular activity in relation to the total cellular LDH activity. To determine total cellular LDH activity, cells were exposed to 0.1% (v/v) Triton X-100 for 30 min at 37°C.

2.7. Quantification of cell proliferation (^{3}H-thymidine incorporation)

After 1 day in culture, cellular DNA synthesis rate was quantified by measuring ^{3}H-thymidine incorporation into cellular DNA, in NTB and DTB cells.

In cells seeded in 24-well plastic cell culture clusters (2 cm^2; diameter 16 mm; TPP) at a cell seeding density of 14×10^5 cells/well, 500 μl of culture medium containing ^{3}H-thymidine (2.5 μCi/ml) was added for 5 h. After this period, the medium was removed and the cells were fixed by incubation with 300 μl of 10% (w/v) trichloroacetic acid (TCA) (1 h at 4°C). Then, cells were washed twice with 10% (w/v) TCA to remove unbound radioactivity, plates were air-dried for 30 min and finally, cells were lysed with 1 mol/L NaOH (280 μl/well). A 250-μl aliquot of the lysate was neutralized with 5 mol/L HCl prior to the addition of scintillation fluid. The radioactivity of the samples was quantified by liquid scintillation counting. Cellular DNA synthesis rate was expressed as incorporation of ^{3}H-thymidine (μCi/mg protein).

2.8. Determination of cell differentiation (alkaline phosphatase activity assay)

Cell differentiation was measured by quantifying cellular alkaline phosphatase (ALP) activity. ALP activity and expression have been used to demonstrate the ability of isolated villous cytotrophoblasts to differentiate into an STB [28].

ALP assays were performed on cells grown for 3 days in 24-well plastic cell culture clusters (2 cm^2; diameter 16 mm; TPP), after seeding at a cell density of 14×10^5 cells/well. The ALP reaction was carried out with 5 mmol/L pNPP as an ALP substrate and 1 mmol/L $MgCl_2$. After 60 min of incubation, the reaction was stopped by adding ice-cold NaOH (0.02 mol/L). The amount of released pNP, reflecting ALP activity, was measured at 405 nm. The results are expressed as nmol pNP min^{-1} mg protein^{-1}.

2.9. Determination of TB cells apoptosis index (TUNEL assay)

A similar amount of NTB and DTB cells (3×10^5) were seeded on glass coverslips for 1 day. To determine the apoptosis index, TUNEL assay (terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick-end labeling) was performed using the In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Briefly, cells were fixed with 4% (w/v) *p*-formaldehyde solution in PBS for 30 min, permeabilized with sodium citrate 0.1% (w/v) and Triton X-100 0.1% (v/v) for 2 min at 4°C and then incubated with fluorescein isothiocyanate-conjugated dUTP for 1 h at 37°C. DAPI (0.5 μg/ml in methanol, 5 min) was used to counter-stain total nuclei.

Table 2
Primer sequences and annealing temperatures used for qRT-PCR

Gene name	Primer sequence (5′ to 3′)	Annealing temperature (°C)
β-Actin	Fwd: AGA GCC TCG CCT TTG CCG AT Rev: CCA TCA CGC CCT GGT GCC T	65
FATP 1	Fwd: CTG CCA TCT GGG AGG AGT TCA Rev: CCG ACC TTG CCG TCC AT	66
FATP 2	Fwd: TAA GCG GAT TGA AGG CAG ATG Rev: GTC CGC AAG GCA AGA GTA GC	66
FATP 4	Fwd: GAC TGC CTC CCC CTC TAC Rev: GAA CTT CTT CCG AAT CAC CAC	65
ACSL 1	Fwd: CGA GGG CGA GGT GTG T Rev: GTG TAA CCA GCC GTC TTT GTC	65

Fwd, forward; Rev, reverse.

Coverslips were mounted on glass slides and visualized under a fluorescence microscope (Nikon 50i, Nikon, Japan). The percentage of TUNEL-stained nuclei was evaluated in relation to 700–2500 DAPI-stained nuclei observed in 15–25 randomly chosen optical fields per slide (in a total of about 4×10^5 nuclei). Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2, UK). Apoptosis index was calculated as the number of apoptotic cells (in % of total cells).

2.10. Protein determination

The protein content of cell monolayers was determined as described by Bradford [29], using BSA as standard.

2.11. Calculations and statistics

For the analysis of the time-course of ^{14}C-AA and ^{14}C-DHA uptake, the parameters of Eq. (1) were fitted to the experimental data by a non-linear regression analysis, using a computer-assisted method [30].

$$A(t) = k_{in}/k_{out}\left(1-e^{-k_{out}\cdot t}\right) \quad (1)$$

$A(t)$ represents the accumulation of ^{14}C-AA or ^{14}C-DHA at time t; k_{in} and k_{out}, the rate constants for inward and outward transport, respectively; and t, the incubation time. A_{max} is defined as the accumulation at steady-state ($t\rightarrow\infty$). k_{in} is given in pmol mg protein^{-1} min^{-1} and k_{out} is given in min^{-1}. In order to obtain clearance values, k_{in} was converted to μl mg protein^{-1} min^{-1}.

For the analysis of the saturation curve of ^{14}C-AA and ^{14}C-DHA uptake, the parameters of the Michaelis–Menten equation were fitted to the experimental data by using a non-linear regression analysis, using a computer-assisted method [30].

Arithmetic means are given with S.E.M. n indicates the total number of replicates obtained from the distinct placentae. Statistical significance of the difference between two groups was analyzed with the Student's t test. Differences were considered to be significant when $P<.05$.

3. Results

3.1. Characteristics of the study groups

As shown in Table 1, control and GDM groups were closely matched in terms of clinical, anthropometrical and demographic characteristics. However, maternal body mass index (BMI) before delivery and fasting blood glucose were significantly higher in the GDM group. Pregnant women of both control and GDM groups had a strict metabolic control as fasting blood glucose and hemoglobin A_{1C} levels were lower than 5 mmol/L and 6%, respectively [15].

3.2. Comparison of characteristics of NTB and DTB cells: viability, proliferation, differentiation and apoptosis

Adequate trophoblast growth, proliferation, differentiation and programmed cell death are crucial to form and maintain the structure and function of the placental epithelium [31]. Alterations in these properties have been associated with pregnancy disorders such as preeclampsia, intrauterine growth restriction and molar pregnancies [31–33]. However, not much is known concerning this subject in GDM. So, we decided to compare viability, proliferation, differentiation and apoptosis of NTB and DTB cells.

Cell viability and differentiation were assessed in NTB and DTB cells after 3 days in culture. These parameters were assessed at this time period because cytotrophoblasts normally differentiate into an STB after 3 days in culture [32]. As can be seen in Fig. 1A and C, cell viability and differentiation were similar in both NTB and DTB cells.

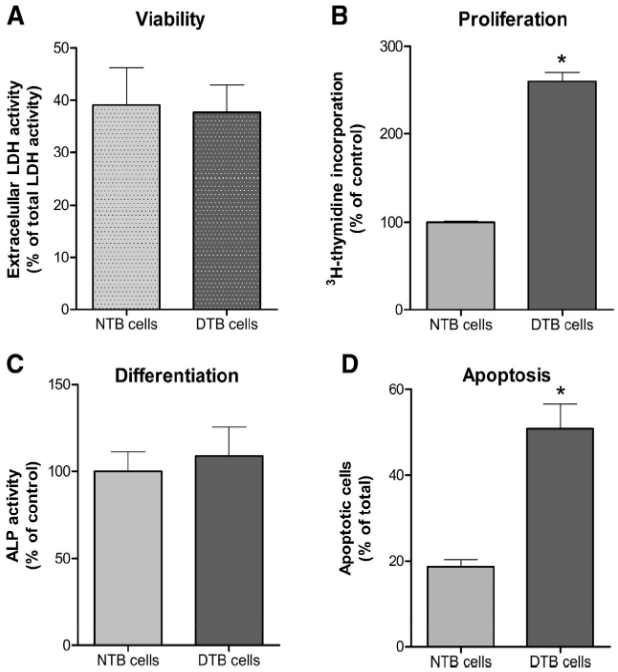

Fig. 1. Viability, proliferation, differentiation and apoptosis in NTB and DTB cells. (A) Viability was determined in cells after 3 days in culture by quantification of extracellular LDH activity (n=18–19, from 3 to 4 distinct placentae). (B) Proliferation was determined in cells after 1 day in culture by quantification of ^{3}H-thymidine incorporation (n=15–16, from 3 distinct placentae). (C) Differentiation was determined in cells after 3 days in culture by quantification of cellular ALP activity (n=14–15, from 3 distinct placentae). (D) Apoptosis index was determined in cells after 1 day in culture by the TUNEL assay (n=7–8, from 3 distinct placentae). Shown are arithmetic means+S.E.M. *Significantly different from NTB cells ($P<.05$) (Student's t test).

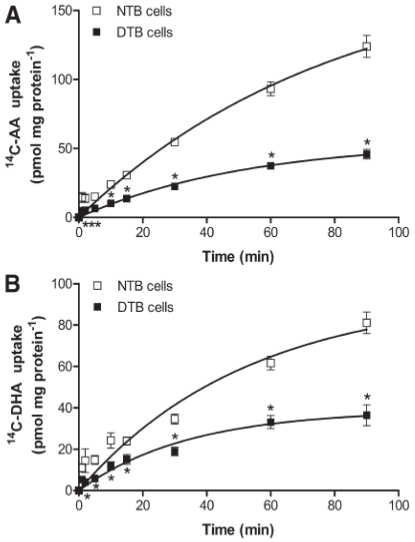

Fig. 2. Time-course of ^{14}C-AA (A) and ^{14}C-DHA (B) uptake by NTB and DTB cells. Cells were incubated at 37°C and pH 7.5, with 100 nmol/L ^{14}C-AA or ^{14}C-DHA for various periods of time (n=6–10, from 3 to 4 distinct placentae). Shown are arithmetic means ±S.E.M. *Significantly different from NTB cells (P<.05) (Student's t test).

Proliferation and apoptosis were analyzed in NTB and DTB cells, after 1 day in culture, since trophoblast proliferation is restricted to cytotrophoblasts [34] and because cytotrophoblasts are more susceptible to apoptosis, induced by certain stimuli, when compared with the STB [35]. DTB cells, when compared with NTB cells, presented a markedly higher (2.6×) proliferation rate, which was accompanied by an increase of the same magnitude (2.7×) in the apoptosis index (Fig. 1B and D). As whole, these results suggest that there is a higher degree of cell turnover in DTB relative to NTB cells.

3.3. Comparison of time-course of ^{14}C-AA and ^{14}C-DHA uptake in NTB and DTB cells

In a second series of experiments, we compared the time-course of accumulation of ^{14}C-AA and ^{14}C-DHA in NTB and DTB cells. For this, cells were incubated with 100 nmol/L ^{14}C-AA or ^{14}C-DHA, pH 7.5, for various periods of time.

As can be seen in Fig. 2, uptake of ^{14}C-AA and ^{14}C-DHA was markedly reduced over time in DTB when compared to NTB cells. In relation to ^{14}C-AA uptake, analysis of the time-course showed that the rate constant of inward transport (k_{in}) was significantly higher in NTB than in DTB cells (22.6±1.8 and 10.5±0.84 µl mg protein^{-1} min^{-1}, respectively), the same happening with the steady-state accumulation (A_{max}) (182.1±24.6 and 55.5±4.5 pmol mg protein^{-1}, respectively); however, no significant differences were found in relation to the rate constant of outward transport (k_{out}) (0.012±0.003 and 0.019±0.003 min^{-1}) (n=6–10) (Fig. 2A). In relation to ^{14}C-DHA uptake, the same pattern of variation was observed for the three parameters: both the k_{in} (18.1±2.0 and 11.4±1.6 µl mg protein^{-1} min^{-1} in NTB and DTB cells, respectively) and the A_{max} (94.9±11.3 and 38.9±3.6 pmol mg protein^{-1} in NTB and DTB cells, respectively) were significantly lower in DTB cells when compared to NTB cells. Again, the k_{out} was unaltered (0.019±0.004 and 0.029±0.006 min^{-1} for NTB and DTB cells, respectively; n=6–10) (Fig. 2B). From these results, we conclude that the efficiency to remove ^{14}C-AA and ^{14}C-DHA from the external medium is significantly lower in DTB when compared to NTB cells.

As can be seen in Fig. 2, uptake of ^{14}C-AA or ^{14}C-DHA by NTB and DTB cells was linear for the first 6 min of incubation. Subsequent experiments were thus performed by using a 6-min incubation period.

3.4. Comparison of kinetics of ^{14}C-AA and ^{14}C-DHA uptake in NTB and DTB cells

In another set of experiments, we compared the initial rates of uptake of ^{14}C-AA and ^{14}C-DHA at increasing substrate concentrations in the apical medium (from 0.05 to 5 µmol/L) in NTB and DTB cells. As can be seen in Fig. 3, uptake of ^{14}C-AA and ^{14}C-DHA for up to 5 µmol/L was linear with increasing substrate concentrations, in both NTB and DTB cells. However, the slope of uptake was significantly higher in NTB than in DTB cells, for both ^{14}C-AA (0.137±0.0019 vs. 0.065±0.0041, respectively; n=7–8) and ^{14}C-DHA (0.089±0.0009 vs. 0.050±0.0007, respectively; n=10–11). Moreover, analysis of uptake of lower concentrations of ^{14}C-AA revealed a saturable component of uptake in both NTB and DTB cells. Although the K_m of uptake was not significantly different between NTB and DTB cells (103±74 and 76±66 nmol/L, respectively), the V_{max} of uptake in DTB cells was about 25% of that of NTB cells (64±19 and 15±6 pmol mg prot^{-1} 6 min^{-1} in NTB and DTB cells, respectively) (Fig. 3A). A saturable component of uptake was also found for lower concentrations of ^{14}C-DHA in NTB cells (K_m of 652±608 nmol/L and V_{max} of 105±75 pmol mg prot^{-1} 6 min^{-1}), but this saturable component of uptake was not found in DTB cells (Fig. 3B).

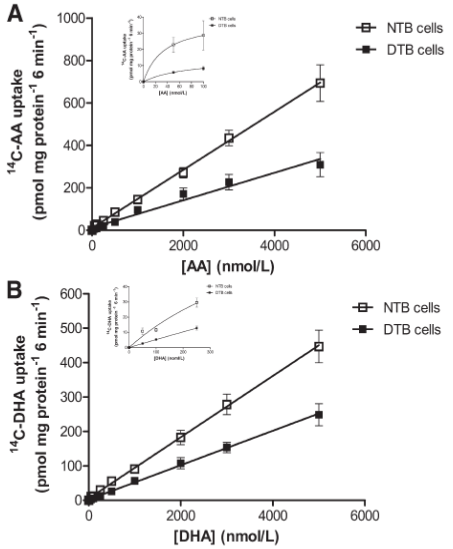

Fig. 3. Kinetics of (A) ^{14}C-AA and (B) ^{14}C-DHA uptake by NTB and DTB cells. (A) Initial rates of uptake were determined in cells incubated at 37°C for 6 min with concentrations of ^{14}C-AA up to 5 µmol/L or 100 nmol/L (inset). (B) Initial rates of uptake were determined in cells incubated at 37°C for 6 min with concentrations of ^{14}C-DHA up to 5 µmol/L or 250 nmol/L (inset) (n=7–11, from 3 to 4 distinct placentae). Shown are arithmetic means±S.E.M.

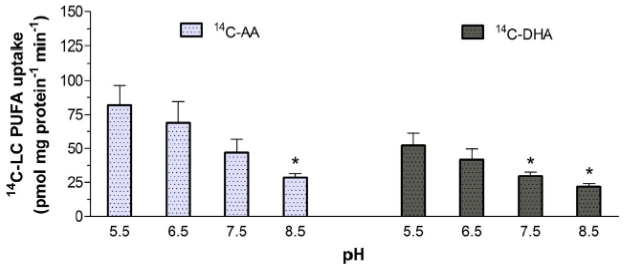

Fig. 4. pH dependence of ^{14}C-AA and ^{14}C-DHA uptake by NTB cells. Initial rates of uptake were determined in cells incubated at 37°C with 500 nmol/L ^{14}C-AA or ^{14}C-DHA for 6 min. The extracellular pH in the preincubation and incubation media ranged from 5.5 to 8.5. Shown are arithmetic means+S.E.M. (n=6, from 2 distinct placentae). *Significantly different from uptake at pH 5.5 (P<.05) (Student's t test).

3.5. Further characterization of ^{14}C-AA and ^{14}C-DHA uptake in NTB cells

3.5.1. pH dependence of uptake

The effect of extracellular pH on the uptake of ^{14}C-AA and ^{14}C-DHA in NTB cells was examined by varying the pH of the preincubation and incubation media from 5.5 to 8.5. These experiments were performed because protonation of long-chain fatty acids facilitates their transport across the membrane lipid bilayer [14]. As shown in Fig. 4, uptake of ^{14}C-AA and ^{14}C-DHA was found to be pH dependent, being higher at acidic pH (5.5).

3.5.2. Specificity of the uptake mechanism

In order to determine the specificity of the membrane transporter(s) involved in ^{14}C-AA and ^{14}C-DHA uptake by NTB cells, we first determined the effect of several long-chain fatty acids (100 μmol/L) upon their uptake by these cells. ^{14}C-AA uptake was not affected by OA (18:1n-9), LA and γ-LNA (18:3n-6). By contrast, palmitic acid (PA; 16:0), eicosapentaenoic acid (EPA; 20:5n-3) and DHA significantly decreased ^{14}C-AA uptake (by 20–35%), with the following ranking order of potency: EPA>DHA=PA (Fig. 5). In relation to ^{14}C-DHA uptake, it was significantly decreased (by 20–40%) by all the fatty acids tested, with the exception of LA. Again, EPA was the most potent of the tested fatty acids (ranking order of potency: EPA>PA=OA=γ-LNA=AA) (Fig. 5).

Placental transport of LC-PUFAs has been described to occur by both a non-protein-mediated mechanism (simple diffusion) and by a protein-mediated mechanism [4,8,9,36]. To study the contribution of these two mechanisms for ^{14}C-AA and ^{14}C-DHA uptake by NTB cells, we first determined the effect of phloretin and DIDS, two non-specific inhibitors of protein-mediated long-chain fatty acid uptake in distinct cell types [37,38]. As shown in Fig. 6, uptake of ^{14}C-AA was slightly decreased by phloretin but increased in the presence of DIDS, and uptake of ^{14}C-DHA was increased by both phloretin and DIDS.

As phloretin and DIDS are non-specific inhibitors of various cellular transport processes [37,38], the role of inhibitors of two specific fatty acid binding proteins, FATP and ACSL, was next determined. Because FATP-mediated uptake of LC-PUFAs was found to be diminished in the face of cellular depletion of ATP [39], we decided to test the effect of ATP depletion (by using dinitrophenol). Dinitrophenol was not able to alter the transport of ^{14}C-AA, and uptake of ^{14}C-DHA was even increased in the presence of this agent (Fig. 6). On the contrary, triacsin C, an inhibitor of the activity of ACSL 1, 3 and 4 [12], was able to markedly reduce ^{14}C-AA and ^{14}C-DHA uptake (to 47% and 63% of control, respectively) (Fig. 6).

These results show that uptake of ^{14}C-AA and ^{14}C-DHA by NTB cells is inhibited by both saturated and unsaturated fatty acids, being however most potently inhibited by other PUFAs (especially by EPA), and that it is dependent on ACSL activity.

3.6. Effect of GDM-associated conditions upon ^{14}C-AA and ^{14}C-DHA uptake by NTB cells

In this series of experiments, we investigated the effect of specific GDM-associated markers, such as elevated concentrations of D-glucose, insulin, leptin and the pro-inflammatory cytokine TNF-α, upon the uptake of ^{14}C-AA and ^{14}C-DHA by NTB cells. As can be seen in Fig. 7, 20 mmol/L D-glucose (corresponding to an hyperglycemic situation [15]) and 100 ng/ml leptin (a concentration known to be representative of hyperleptinemia in GDM [40]) were devoid of effect upon ^{14}C-AA and ^{14}C-DHA uptake. In contrast, high concentrations of

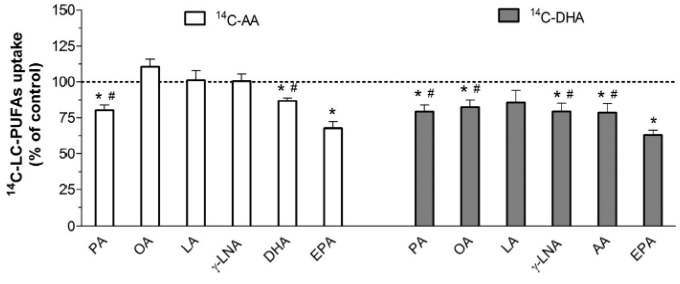

Fig. 5. Specificity of ^{14}C-AA and ^{14}C-DHA uptake by NTB cells. Initial rates of uptake were determined in cells incubated at 37°C with 500 nmol/L ^{14}C-AA or ^{14}C-DHA for 6 min. Cells were both preincubated for 20 min and incubated with ^{14}C-AA or ^{14}C-DHA in the absence (control; corresponding to 100%) or presence of PA, OA, LA, γ-LNA, DHA, AA or EPA, all at 100 μmol/L (n=6–12, from 3 to 4 distinct placentae). Shown are arithmetic means+S.E.M. *Significantly different from control (P<.05); #Significantly different from EPA (Student's t test).

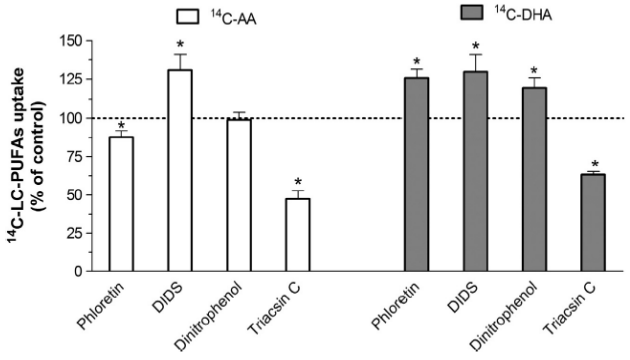

Fig. 6. Pharmacological characterization of ^{14}C-AA and ^{14}C-DHA uptake by NTB cells. Initial rates of uptake were determined in cells incubated at 37°C with 500 nmol/L ^{14}C-AA or ^{14}C-DHA for 6 min. Cells were both preincubated for 20 min and incubated with ^{14}C-AA or ^{14}C-DHA in the absence (control; corresponding to 100%) or presence of 0.5 mmol/L phloretin (Phloretin), 0.5 mmol/L DIDS (DIDS), 1 mmol/L dinitrophenol (Dinitrophenol) or 10 μmol/L triacsin C (Triacsin C) (n=8–14 from 4 to 5 distinct placentae). Shown are arithmetic means+S.E.M. *Significantly different from control (P<.05) (Student's t test).

TNF-α (100 ng/L), known to be representative of the increased plasma levels of inflammation observed in GDM [41], increased by a similar magnitude (20%) the uptake of both LC-PUFAs. Moreover, insulin at 50 nmol/L, corresponding to hyperinsulinaemia [40] increased ^{14}C-DHA uptake by 23% (Fig. 7).

3.7. Comparison of mRNA expression levels of AA and DHA transporters in NTB and DTB cells

Because uptake of ^{14}C-AA and ^{14}C-DHA is significantly reduced in DTB cells in relation to NTB cells, we decided to compare the mRNA levels of LC-PUFAs transporter/binding proteins in NTB and DTB cells, by qRT-PCR.

pFABPpm is a preferential transporter for LC-PUFAs, particularly DHA and AA, at the placental level [3,8]. FATP 1, 2 and 4 have also been implicated in the uptake of LC-PUFAs [3], and these three isoforms are present in the placenta [4,36,42]. On the contrary, FAT/CD36 does not seem to be of major importance for placental transport of DHA and AA [3]. Additionally, intracellular ACSLs are also known to stimulate fatty acid uptake, and recently, exposure to LC-PUFAs was found to increase the uptake of LC-PUFAs in a human trophoblast cell line by increasing the activity and expression of ACSL, particularly ACSL 1 [8].

So, we quantified the mRNA expression of FATP 1, FATP 2, FATP 4 and ACSL 1. Despite the recognized importance of pFABPpm for placental transport of DHA and AA, its complete cDNA or amino acid sequence has not been reported [3]. As a consequence, we could not quantify the mRNA levels of this transporter.

As shown in Fig. 8, the mRNA levels of FATP 1, FATP 2 and FATP 4 were not significantly different in NTB and DTB cells. On the other hand, ACSL 1 mRNA levels were decreased by almost 50% in DTB relative to NTB cells.

4. Discussion

The aim of this study was to characterize the uptake of AA and DHA by normal human trophoblasts and to investigate if uptake of these LC-PUFAs is altered in GDM and in response to some of its associated conditions (increased levels of glucose, insulin, leptin and TNF-α). For this, we compared ^{14}C-AA and ^{14}C-DHA uptake by NTB and DTB cells and tested the effect of specific GDM-associated conditions upon the uptake of these LC-PUFAs by NTB cells.

Comparison of NTB and DTB cells showed a marked increase (to 2.6–2.7×) in proliferation and apoptosis index in DTB, in relation to NTB cells, suggesting that a GDM-related phenotype — a higher cell turnover — may be preserved in DTB cells. In line with this, Sgarbosa et al. also reported an increase in trophoblast apoptosis index in GDM, suggesting that hyperglycemia may be a key factor evoking this response [43]. Interestingly, in other pregnancy

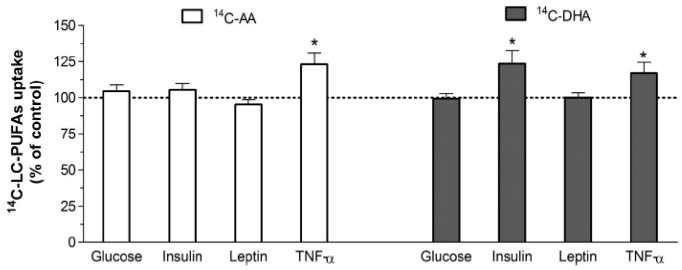

Fig. 7. Effect of GDM-associated conditions on ^{14}C-AA and ^{14}C-DHA uptake by NTB cells. Initial rates of uptake were determined in cells incubated at 37°C with 500 nmol/L ^{14}C-AA or ^{14}C-DHA for 6 min. Cells were both preincubated for 20 min and incubated with ^{14}C-AA or ^{14}C-DHA in the absence (control; corresponding to 100%) or presence of 20 mmol/L D-glucose (Glucose), 50 nmol/L insulin (Insulin), 100 ng/ml leptin (Leptin) or 100 ng/L TNF-α (TNF-α) (n=8–9, from 3 distinct placentae). Shown are arithmetic means+S.E.M. *Significantly different from control (P<.05) (Student's t test).

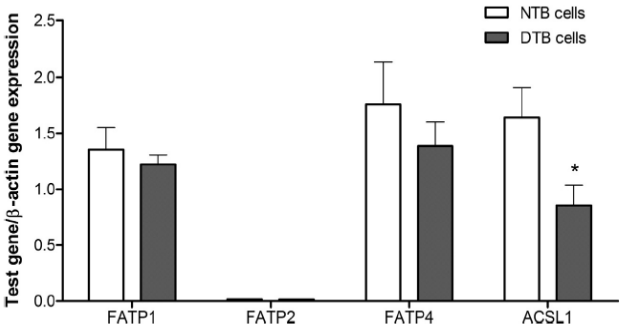

Fig. 8. Comparison of mRNA levels of fatty acid transport protein 1 (FATP 1), 2 (FATP 2) and 4 (FATP 4) and ACSL 1 in NTB and DTB cells, by qRT-PCR (n= 6 placentae for each group). Shown are arithmetic means+S.E.M. corresponding to the expression of each test gene relative to β-actin. *Significantly different from NTB cells (P<.05) (Student's t test).

pathologies, the turnover of trophoblasts cells is similarly altered as reported by us for GDM: an up-regulation of proliferation in preeclampsia and molar pregnancies [33] and an up-regulation of apoptosis in preeclampsia, intrauterine growth restriction and molar pregnancies [32,44].

The characteristics of ^{14}C-AA and ^{14}C-DHA uptake by NTB cells are compatible with the involvement of a protein-mediated mechanism, as demonstrated by the following characteristics: the presence of saturable kinetics, the sensitivity to the non-specific inhibitor of plasma membrane fatty acid transporters, phloretin, and the inhibitory effect of long-chain fatty acids, particularly LC-PUFAs. LC-PUFAs uptake in skeletal muscle cells [38], myocytes [45] and hepatocytes [37] showed a higher sensitivity to non-specific inhibitors of membrane fatty acid transporters, when compared to NTB cells. It thus appears that LC-PUFAs uptake in NTB cells is less dependent on protein-mediated mechanisms, when compared to other tissues. Moreover, the ATP independence of ^{14}C-AA and ^{14}C-DHA uptake argues against the involvement of FATP and supports the previous notion that fatty acid uptake is not dependent on cellular ATP [9]. Importantly, uptake of ^{14}C-AA and ^{14}C-DHA by NTB cells was found to greatly depend on the activity of ACSLs, as it was markedly inhibited by triacsin C. So, inhibition of ACSL-mediated fatty acid esterification into acyl-CoA can modulate, to a great extent, the degree of transport of ^{14}C-AA and ^{14}C-DHA into trophoblasts. This observation is in good agreement with recent data obtained in another cellular model of human trophoblasts, the Bewo cells [8,12]. Nevertheless, because ^{14}C-AA and ^{14}C-DHA uptake by NTB cells was strongly (40–50%) but not completely inhibited by triacsin C, we cannot rule out the possible involvement of other transporters/binding proteins in this process. This transporter may well correspond to pFABPpm, which was found on the microvillous membrane of the STB [36], has a preferential affinity and binding capacity for AA and DHA and is triacsin C-insensitive [4,11,46].

Kinetic analysis showed that, besides a saturable component (for lower substrate concentrations), a non-saturable component (for higher substrate concentrations) was also involved in ^{14}C-AA and ^{14}C-DHA uptake. So, protein-mediated transport is the more important mechanism for uptake of lower concentrations of these LC-PUFAs and, distinctly, simple diffusion is quantitatively the more important mechanism for uptake at higher concentrations of these compounds. This observation is in agreement with claims of other authors suggesting that, under blood physiological concentrations of LC-PUFAs (nanomolar range), most of the cellular uptake occurs via the protein-mediated pathway, simple diffusion being quantitatively less important [47]. Nevertheless, the observed increase of ^{14}C-AA and ^{14}C-DHA uptake at an acidic pH, where protonation of the LC-PUFAs increases, is most probably due to an increase in passive diffusion transport across the cell membrane [14].

Human type 2 diabetes and animal models of insulin resistance or type 2 diabetes are associated with changes in LC-PUFAs uptake in several tissues [48]. However, not much is known concerning the effect of GDM on the placental uptake of LC-PUFAs. In the present study, we show that uptake of ^{14}C-AA and ^{14}C-DHA is markedly reduced in DTB cells, through a decrease in both the saturable and the non-saturable components of uptake. In relation to the saturable component, a decrease in the V_{max} of transporter-mediated uptake of ^{14}C-AA was observed, and for ^{14}C-DHA, the transporter-mediated component could not even be found.

Moreover, qRT-PCR analysis revealed that mRNA levels of FATP 1, 2 and 4 were not significantly changed in DTB cells, but importantly, a 50% reduction in the mRNA levels of ACSL 1 in DTB cells was found. This observation further supports the involvement of ACSL 1 in ^{14}C-AA and ^{14}C-DHA uptake by human trophoblasts and clearly implicates the decrease in its expression as related to the decrease in ^{14}C-AA and ^{14}C-DHA uptake found in DTB cells. Of note, the interaction between FATP 1 and ACSL 1 activity, which is still a matter of debate [47], does not appear to exist in trophoblast cells.

By clearly showing a decrease in the placental transport of LC-PUFAs in GDM associated with a decrease in ACSL1 mRNA expression levels, our results may well explain the reduced plasma levels of AA and DHA found in neonates born from women with GDM [20,21]. This is very important in the context of the known crucial role that LC-PUFAs have for fetal visual, behavioral and cognitive development [3,4].

Not much is known concerning the effect of GDM-associated conditions upon the placental uptake of fatty acids [49–51]. In tissues other than the placenta, insulin and leptin were found to increase the functional expression of fatty acid transporters or binding proteins [48]. So, we decided also to evaluate the short-term effect of high glucose, insulin, leptin and TNF-α upon ^{14}C-AA and ^{14}C-DHA uptake by NTB cells. Glucose and leptin showed no effect, but TNF-α caused a 20% increase in ^{14}C-AA uptake, and both insulin and TNF-α increased ^{14}C-DHA uptake by 20–25%. In line with our results, others found out that TNF-α induces accumulation of fatty acids in human trophoblasts by up-regulating the expression of phospholipase A_2 [52] and fatty acid synthase [53]. By contrast, TNF-α was recently found to have no effect on fatty acid accumulation by NTB cells [49], but the different TNF-α concentrations used by Lager et al. may explain this discrepancy. In relation to insulin, it was previously reported to be devoid of effect upon AA and DHA uptake in Bewo cells [51] and upon the uptake of OA in NTB cells [50]. The lower insulin concentrations used in these studies (0.1–10 nmol/L) might not be sufficient to elicit

an increase in ^{14}C-DHA uptake as observed by us. Interestingly enough, insulin has a relatively rapid stimulatory effect upon fatty acid uptake in other tissues (e.g., cardiac and skeletal muscle and adipocytes) [48,54].

Concerning the results obtained with leptin, which did not affect ^{14}C-AA and ^{14}C-DHA uptake by NTB cells, they perfectly agree with a previous report [51], showing that long-chain fatty acids stimulate leptin internalization in trophoblasts, thereby interfering with its binding to the receptors. In relation to the effect of high glucose levels upon the placental uptake of LC-PUFAs, almost nothing is known [55]. Available data state that chronic hyperglycemic conditions, in the presence of a mixture of fatty acids, reduce the expression of adipophilin in NTB cells [55]. However, our results clearly show that short periods of hyperglycaemia do not seem to have a great impact upon ACSL-mediated placental uptake of AA and DHA.

An important point to discuss is the contrasting effect of acute insulin and TNF-α upon LC-PUFAs uptake (increase) and the observed changes in the uptake of these fatty acids in DTB cells (decrease). These results may imply that either (a) the acute and chronic effect of GDM and its conditions is different because, e.g., of chronic adaptative changes in the mRNA and protein expression levels of specific transporters or (b) changes in LC-PUFA uptake in DTB cells cannot be attributed to changes in the level of a single condition, but rather result from simultaneous and interacting long-term changes in several metabolic mediators.

In conclusion, this study shows that uptake of ^{14}C-AA and ^{14}C-DHA by NTB cells involves both a protein-mediated mechanism, for lower concentrations, and simple diffusion, for higher concentrations, and that it is highly dependent on ACSL 1 activity. In DTB cells, uptake of both ^{14}C-AA and ^{14}C-DHA is markedly inhibited, which is associated with a marked decrease in ACSL 1 gene expression. By contrast, short-term exposure to some GDM-associated conditions (insulin and TNF-α) produced a 20% increase in LC-PUFA uptake by NTB cells. Finally, DTB cells show an increased cell turnover (increased proliferation and apoptosis) when compared with NTB. In view of the important role of LC-PUFAs during pregnancy, the marked decrease in LC-PUFAs placental uptake associated with GDM may have deleterious consequences for fetal and postnatal development.

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Assessment of a GDM-associated hallmark (oxidative stress) at placental level

The information contained in this chapter is included in the following original publication:

D. Araújo JR, Ramalho C, Correia-Branco A, Faria A, Ferraz T, Keating E, Martel F. Parallel increase in placental oxidative stress and antioxidant defenses occurs in type 1 but not gestational diabetes.

Placenta. 2013;34:1095-1098.
DOI: 10.1016/j.placenta.2013.09.001.
IF: 3.12

D. Parallel increase in placental oxidative stress and antioxidant defenses occurs in type 1 but not gestational diabetes

Placenta 34 (2013) 1095–1098

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Short communication

A parallel increase in placental oxidative stress and antioxidant defenses occurs in pre-gestational type 1 but not gestational diabetes

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ARTICLE INFO

Article history:
Accepted 2 September 2013

Keywords:
Maternal diabetes
Oxidative stress
Placenta

ABSTRACT

We aimed to determine the oxidative stress status in placentas obtained from gestational (GDM) and type 1 (T1D) diabetic pregnancies. Malonaldehyde and protein carbonyls, two biomarkers of oxidative damage, were higher in T1D but not in GDM placentas. Also, higher reduced glutathione and lower oxidized glutathione levels and higher glutathione peroxidase activity were found in T1D but not in GDM placentas. These results suggest that T1D placentas may develop a protective antioxidant mechanism to overcome higher oxidative stress levels.

1. Introduction

Diabetes is the most prevalent metabolic disorder diagnosed in pregnant women [1,2]. Both gestational (GDM) and pre-gestational type 1 diabetes (T1D) are associated with an increased risk of adverse perinatal outcomes and later in life metabolic diseases for both the mother and the offspring [2,3]. While maternal hyperglycemia is recognized as the main factor responsible for those complications [3,4], other factors, such as oxidative stress, also seem to play an important role in the pathophysiology of maternal diabetes and its complications [3]. Increased levels of oxidative stress have been found in diabetic pregnant women. However, the majority of these studies analyzed maternal tissues such as plasma and serum [5–7], and only a few analyzed both GDM and T1D human placentas [8].

So, in order to test the hypothesis that diabetic pregnancy is associated with an increase in placental oxidative stress, we decided to compare the placental oxidative stress status in uncomplicated, GDM and T1D pregnancies.

2. Materials and methods

Following ethical approval, human placentas were collected at the Department of Obstetrics and Gynecology of Centro Hospitalar S. João, Porto, from uncomplicated (control), GDM and T1D singleton term pregnancies (Table 1), within half an hour after spontaneous delivery or elective cesarean section. GDM was diagnosed according to published criteria defined by the International Association of the Diabetes and Pregnancy Study Group (IADPSG) consensus panel [9]. None of these pregnancies were associated with any major maternal or fetal pathology or diabetes-associated complications.

Placental villous tissue homogenates were incubated in the absence or presence of *tert*-butyl hydroperoxide (*tert*-BOOH) 3 mM for 1 h at 37 °C, as previously described [10]. Afterwards, levels of malonaldehyde (MDA), protein carbonyls, total (GSX), oxidized (GSSG) and reduced (GSH) glutathione and the activity of Se-dependent glutathione peroxidase (GPX) were quantified in supernatants as described elsewhere [10,11].

Statistical significance ($P < 0.05$) between various groups and two groups were analyzed by one-way ANOVA (followed by the Bonferroni test) and Student's *t* test, respectively. Pearson correlation coefficients were calculated to determine significant associations between maternal metabolic parameters and oxidative stress.

3. Results and discussion

Biomarkers of oxidative damage to lipids (MDA) and proteins (carbonyls) [12] were elevated in T1D, but not in GDM placentas, when compared to control (Fig. 1a and b), indicating that greater lipid and protein oxidation levels exists in T1D placentas only. Exposure to an oxidative challenge (*tert*-BOOH) induced an increase in MDA levels in all groups, although the relative increase was smaller in T1D (2×) than in control and GDM placentas (9 and 11×, respectively) (Fig. 1a). By contrast, protein carbonyl levels were not changed by *tert*-BOOH (Fig. 1b).

Hyperglycemia is one of the most important mechanisms leading to increased oxidative stress in GDM [1,5,13]. However, no correlations were found between placental biomarkers of oxidative stress and maternal third trimester fasting glycemia or glycosylated

Abbreviations: GDM, gestational diabetic pregnancies; GSX, total glutathione; MDA, malonaldehyde; T1D, pre-gestational type 1 diabetic pregnancies; *tert*-BOOH, *tert*-butyl hydroperoxide.

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0143-4004/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.placenta.2013.09.001

Table 1
Clinical characteristics of the three study groups.

	Control	GDM[a]	T1D[b]
Mothers			
N	11	15	5
Maternal age (years)	33.1 ± 1.3	34.9 ± 0.9	27.2 ± 2.9*#
BMI before delivery (Kg/m^2)[c]	26.5 ± 1.6	31.6 ± 1.0*	23.7 ± 1.4#
Gravida (*n*)	2.4 ± 0.4	2.3 ± 0.2	1.8 ± 0.6
Parity (*n*)	0.9 ± 0.3	1.3 ± 0.2	0.6 ± 0.4
Mode of delivery			
Vaginal [*n* (%)]	4 (36)	6 (40)	2 (40)
Cesarean [*n* (%)][d]	7 (64)	9 (60)	3 (60)
Therapeutics of GDM [*n* (%)]	–	No insulin: 8 (53) – Insulin[e]: 7 (47)	
Fasting blood glucose (mM)[f]			
All	3.9 ± 0.1	4.8 ± 0.2*	7.9 ± 1.6*#
Insulin therapy		4.4 ± 0.3	
No insulin therapy		5.0 ± 0.2*	
HbA_{1C} (%)[g]			
All	–	5.6 ± 0.1	6.3 ± 0.4
Insulin therapy		5.6 ± 0.2	
No insulin therapy		5.7 ± 0.2	
Periconceptional FA use [*n* (%)][h]	10 (90)[j]	14 (93)[j]	3 (60)[j]
Smokers [*n* (%)][j]	0 (0)	0 (0)	1 (20%)
Infants			
Gestational age at birth (weeks)[k]	39.6 ± 0.3	39.2 ± 0.2	37.6 ± 0.7*#
Birth weight (g)[l]	3200 ± 128	3486 ± 138	3293 ± 166
Length (cm)[m]	48.2 ± 0.5	49.6 ± 0.4*	47.9 ± 1.0
SGA newborn [*n* (%)][n]	1 (9)	0 (0)	0 (0)
AGA newborn [*n* (%)]	9 (82)	11 (73)	5 (100)
LGA newborn [*n* (%)]	1 (7)	4 (29)	0 (0)
Placental weight (g)	621.7 ± 49.6	702.3 ± 41.9	588.0 ± 48.9
Sex [*n* (%)]	Male: 2 (18) Female: 9 (82)	Male: 7 (47) Female: 8 (53)	Male: 3 (60) Female: 2 (40)
5-min Apgar Score	9.5 ± 0.2	9.3 ± 0.2	10.0 ± 0.0#

Values represent mean ± SEM.
*Significantly different from control ($P < 0.05$); #significantly different from GDM ($P < 0.05$).
[a] GDM, gestational diabetic pregnancies.
[b] T1D, pre-gestational type 1 diabetic pregnancies.
[c] BMI, body mass index.
[d] All cesarean sections were elective except one from control and one from GDM group, which were laboring.
[e] The criteria for initiating insulin therapy was the presence of a fasting glycemia ≥90 mg/dL (5 mM) or a 2 h-postprandial blood glucose level ≥120 mg/dL (6.7 mM), despite consistent dietary and exercise adjustments.
[f] The majority of values were obtained at 24–28 weeks of gestation. Parameter unknown for 3 subjects from GDM group and 1 from T1D group. Women with T1D were treated with intermediate or long-acting insulin regimens in order to achieve a fasting glycemia <90 mg/dL and a 2 h-postprandial blood glucose level <120 mg/dL.
[g] Values obtained at 35–36 weeks of gestation. Parameter unknown for one subject from GDM group and for all subjects from control group, as this assay is not typically ordered for subjects with no history of glucose mismanagement.
[h] FA, folic acid. Dosage and initiation period unknown.
[i] Parameter unknown for 1 subject.
[j] Parameter unknown for 2 subjects from control group and 1 from T1D group.
[k] Gestational age: number of completed weeks at the time of delivery, determined by prenatal ultrasound at 11–13 weeks.
[l] Birth weight was evaluated to the nearest gram.
[m] Length was evaluated to the nearest tenth of a centimeter after birth.
[n] SGA, small-for-gestational-age; AGA, adequate-for-gestational-age; LGA, large-for-gestational-age, classified according published reference standards (Battaglia FC, Lubchenco LO (1967) A practical classification of newborn infants by weight and gestational age. J Pediatr 71:159–163).

hemoglobin (HbA_{1C}) in GDM pregnancies (which do not present overt hyperglycemia at this time point) (Table 1). Also, no correlations were observed between biomarkers of oxidative stress, body mass index and gestational or maternal age in GDM women (results not shown). Due to the low number of T1D placentas analyzed ($n = 5$), correlation data are not shown.

Glutathione and GPX are reliable indicators of antioxidant status [13]. When compared to control and GDM placentas, higher GSH and lower GSSG concentrations (Fig. 2b and c) and a higher [GSH]/[GSSG] ratio (78 ± 45 for T1D, 1.3 ± 0.4 for GDM, and 0.8 ± 0.3 for

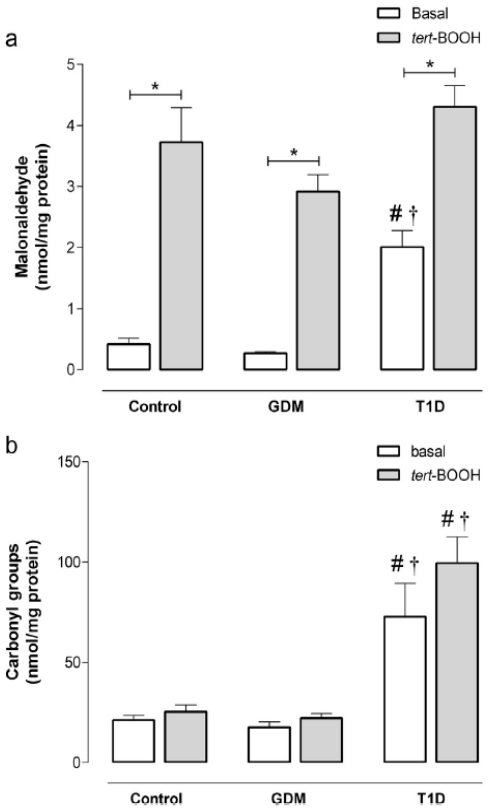

Fig. 1. Levels of malonaldehyde (a) and protein carbonyls (b) in placentas from control ($n = 11$), gestational diabetic (GDM; $n = 15$) and pre-gestational type 1 diabetic (T1D; $n = 5$) pregnancies. These parameters were determined in human placental homogenates after a 1 h-exposure to 3 mM *tert*-butyl hydroperoxide (*tert*-BOOH) or its solvent (basal). Shown are arithmetic means + S.E.M. #Significantly different from control placentas with the same treatment ($P < 0.05$). †Significantly different from GDM placentas with the same treatment ($P < 0.05$). *Significantly different from basal ($P < 0.05$).

control) were found in T1D placentas. On the other hand, GSX, GSH and GSSG levels and [GSH]/[GSSG] ratios were similar in GDM and control placentas (Fig. 2a–c). In the presence of *tert*-BOOH, the decrease in GSH levels was greater in T1D than in control and GDM placentas, suggesting that T1D glutathione is more sensitive to an additional oxidative challenge (Fig. 2b). Additionally, GPX basal activity was higher in T1D but not in GDM placentas in comparison to control, but exposure to *tert*-BOOH decreased the enzyme activity to similar levels in all groups (Fig. 2d).

As a whole, these results suggest that a compensatory antioxidant mechanism – upregulation of glutathione/GPX system – may develop in T1D placentas to overcome higher oxidative stress levels. In agreement with our results, a parallel increase in blood levels of oxidative stress biomarkers and antioxidant enzymes activity have been reported in T1D pregnant women [14,15].

Concerning GDM, we did not find any effect on placental oxidative stress and antioxidants levels, even after stratification for insulin and non-insulin therapy and for labored (vaginal) or non-labored (elective cesarean section) deliveries (results not shown). By contrast, in controls, levels of MDA were more sensitive to *tert*-

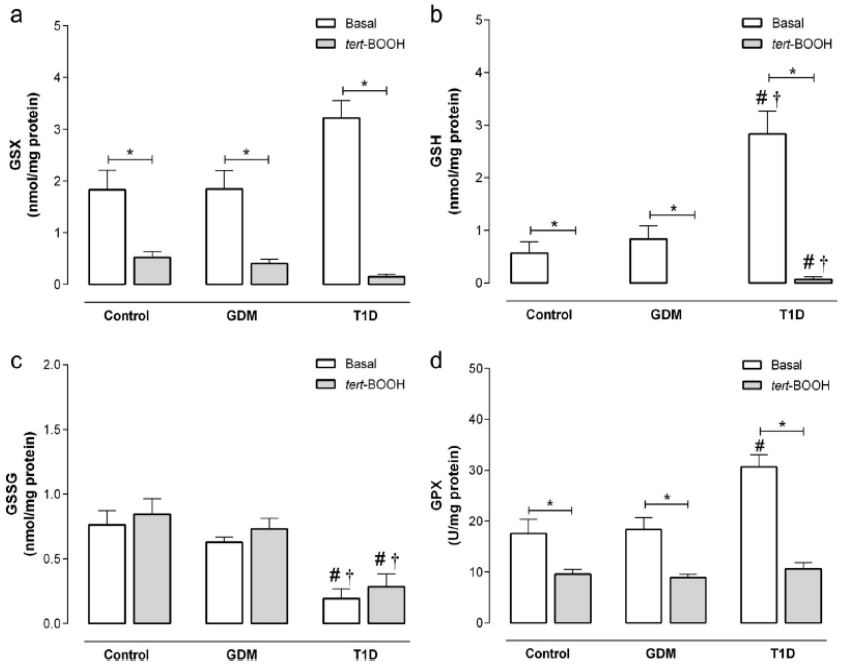

Fig. 2. Total (GSX) (a), oxidized (GSSG) (b) and reduced (GSH) glutathione (c) levels, and glutathione peroxidase activity (GPX) (d) in placentas from control ($n = 8–11$), gestational diabetic (GDM; $n = 12–15$) and pre-gestational type 1 diabetic (T1D; $n = 5$) pregnancies. These parameters were determined in human placental homogenates after a 1 h-exposure to 3 mM *tert*-butyl hydroperoxide (*tert*-BOOH) or its solvent (basal). Shown are arithmetic means + S.E.M. #Significantly different from control placentas with the same treatment ($P < 0.05$). †Significantly different from GDM placentas with the same treatment ($P < 0.05$). *Significantly different from basal ($P < 0.05$).

BOOH and basal GPX activity was lower in labored than in non-labored placentas (results not shown). So, normal labored deliveries appear to be more susceptible to placental oxidative stress than non-labored deliveries, reinforcing a previous conclusion by Cindrova-Davies et al. [16].

In contrast to our results, previous studies have associated GDM with increased oxidative stress [7,17–19]. We believe that the main reason for this apparent inconsistency is that women in our GDM group were diagnosed according to the new IADPSG criteria [9], which encompass women with a less severe diabetic phenotype. This is supported by the observation of HbA_{1C} levels at 35–36 weeks of gestation in GDM women within the acceptable range for managed diabetes (≤5.7%) (Table 1) [20,21].

In conclusion, increased levels of oxidative stress are associated with an up-regulation, and higher sensitivity to oxidative insults, of the antioxidant glutathione system in T1D but not in GDM placentas.

Acknowledgments

This work was financially supported by Fundação para a Ciência e a Tecnologia (FCT) and COMPETE, QREN and FEDER (PTDC/SAU-OSM/102239/2008, SFRH/BD/63086/2009 and SFRH/BPD/40170/2007).

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CHAPTER III

Modulation of placental nutrient transport by GDM-associated hallmarks

The information contained in this chapter is included in the following original publications:

E. Araújo JR, Gonçalves P, Martel F. Modulation of glucose uptake in a human choriocarcinoma cell line (BeWo) by dietary bioactive compounds and drugs of abuse.

J Biochem. 2008;144:177-186
DOI: 10.1093/jb/mvn054.
IF: 2.72

F. Araújo JR, Pereira AC, Correia-Branco A, Keating E, Martel F. Oxidative stress induced by *tert*-butylhydroperoxide interferes with the placental transport of glucose: *in vitro* studies with BeWo cells.

Eur J Pharmacol. *In press*
DOI: 10.1016/j.ejphar.2013.10.023.
IF: 2.59

G. Araújo JR, Correia-Branco A, Pereira AC, Pinho MJ, Keating E, Martel F. Oxidative stress decreases uptake of neutral amino acids in a human placental cell line (BeWo cells).

Reprod Toxicol. 2013;40:76-81
DOI: 10.1016/j.reprotox.2013.06.073.
IF: 3.14

A. Araújo JR, Correia-Branco A, Moreira L, Ramalho C, Martel F, Keating E. Folic acid uptake by the human syncytiotrophoblast is affected by gestational diabetes, hyperleptinemia, and TNF-α. Pediatr Res. 2013;73:388-394 (please see pages 29-35).

B. Araújo JR, Correia-Branco A, Ramalho C, Gonçalves P, Pinho MJ, Keating E, Martel F. L-methionine placental uptake: characterization and modulation in gestational diabetes mellitus. Reprod Sci. 2013;20:1492-1507 (please see pages 37-52).

C. Araújo JR, Correia-Branco A, Ramalho C, Keating E, Martel F. Gestational diabetes mellitus decreases placental uptake of long-chain polyunsaturated fatty acids: involvement of long-chain acyl-CoA synthetase. J Nutr Biochem. 2013;24:1741-1750 (please see pages 53-62).

J. Biochem. **144,** 177–186 (2008)
doi:10.1093/jb/mvn054

Modulation of Glucose Uptake in a Human Choriocarcinoma Cell Line (BeWo) by Dietary Bioactive Compounds and Drugs of Abuse

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Received February 8, 2008; accepted April 11, 2008; published online April 19, 2008

The aim of this work was to investigate the putative modulation of glucose uptake in trophoblast cells by several dietary compounds and by drugs of abuse. For this, the acute (26 min) and chronic (48 h) effect of these substances on the apical uptake of ^{3}H-2-deoxy-D-glucose (^{3}H-DG) by a human choriocarcinoma cell line (BeWo) was determined. ^{3}H-DG apical uptake by BeWo cells was time dependent, displayed saturable kinetics ($V_{max} = 1210 \pm 29$ nmol mg protein^{-1} 6 min^{-1} and $K_m = 13.4 \pm 0.5$ mM) and was insulin-insensitive and cytochalasin B-sensitive (by up to 60%). Acutely, acetaldehyde (30–100 mM), resveratrol, xanthohumol, epigallocatechin-3-gallate (100 μM), chrysin and quercetin (10–100 μM) decreased ^{3}H-DG apical uptake, whereas rutin, catechin (10–100 μM), epicatechin (100 μM) and ethanol (10 mM) increased it. Quercetin and xanthohumol seem to be non-competitive inhibitors of ^{3}H-DG apical uptake, whereas both epigallocatechin-3-gallate and acetaldehyde decreased both the K_m and V_{max} values. Chronically, rutin and myricetin increased the apical uptake of ^{3}H-DG both isolated (0.1–1 μM) and in combination (both at 1 μM), whereas theophylline (0.1–1 μM) and amphetamine, 3,4-methylenedioxymethamphetamine (0.25–1 μM) and Δ^{9}-tetrahydrocannabinol (1 nM) decreased it. In conclusion, ^{3}H-DG apical uptake by BeWo cells is differentially modulated by different compounds present in drinks and by drugs of abuse.

Key words: BeWo cells, drugs of abuse, glucose uptake, methylxanthines, polyphenols.

Abbreviations: DG, 2-deoxy-D-glucose; EGCG, epigallocatechin-3-gallate; GLUT, facilitative glucose family of transporters; IGF-1, insulin growth factor-1; MDMA, 3,4-methylenedioxymetamphetamine; SGLT1, sodium-glucose co-transporter 1; THC, tetrahydrocannabinol.

Glucose serves as the primary source of energy for metabolism and growth of the feto-placental unit, and thus the supply of glucose from maternal blood to fetal circulation represents a major determinant of fetal growth and development (*1*, *2*). Glucose supply to the fetus is dependent on placental glucose transport from the maternal circulation. At the placental level, glucose transport is thought to be mediated by one or more members of the facilitative glucose family of transporters (GLUTs). This assumption is largely based on the characteristics of placental glucose transport. Transport of glucose in both the microvillous and basal membrane of the syncytiotrophoblast has similar kinetic characteristics, is sodium-independent, selective for D- over L-glucose and sensitive to inhibition by phloretin and cytochalasin B (*3–5*).

The GLUT1 glucose transporter, present at both the microvillous and basal membranes of the syncytial barrier, is the predominant glucose transporter expressed in the placenta (*6–10*), and the primary isoform involved in the transplacental movement of glucose. The distribution of GLUT1 within the syncytiotrophoblast is asymmetric, with a greater degree of expression at the microvillous membrane than at the basal membrane. Apart from GLUT1, GLUT3 mRNA was also reported to be expressed in the human placenta (*11*), but GLUT3 protein appears not to be expressed in the syncytiotrophoblast layer of the placenta (*8*, *12*, *13*), but rather in the arterial vascular endothelium (*14*). Preliminary results also suggest that GLUT2 and GLUT5 may be expressed in the apical and basal membranes of the human placenta, respectively (*15*), but these findings remain unsubstantiated.

Although the existence and nature of the glucose transporters in the placenta have been known for many years, there is little data on the expression and activity of glucose transporters in pathological conditions (*13*, *16*, *17*). Also, little is known about glucose transport regulation in the placenta other than the effects of hyper and hypoglycaemia and of insulin (and IGF-1) (*18*).

Pregnant women are frequently exposed to xenobiotics due to lifestyle factors such as diet, smoking, drug abuse and alcohol consumption. It is known that these conditions have (or may have) deleterious effects on the fetus, but the cellular mechanisms involved remain to be completely elucidated. So, we decided to investigate the putative modulation of glucose uptake in trophoblast cells by some dietary bioactive compounds and by some drugs of abuse, by determining the acute and chronic effect of these substances upon the apical uptake of ^{3}H-2-deoxy-D-glucose (^{3}H-DG) by BeWo cells. The substances tested were ethanol (and its metabolite

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acetaldehyde), some polyphenolic compounds (catechin, chrysin, epicatechin, epigallocatechin-3-gallate, myricetin, quercetin, resveratrol, rutin and xanthohumol) present in alcoholic (*e.g.* red wine) or non-alcoholic drinks (*e.g.* green tea), two methylxanthines (caffeine and teophylline) present in drinks such as coffee and tea, and the drugs of abuse amphetamine, *ecstasy* (MDMA), tetrahydrocannabinol (THC), nicotine and cocaine.

The BeWo cell line derives from a human gestational choriocarcinoma, and is a known cellular model of the human syncytiotrophoblast (*19*, *20*), having been much used to investigate placental trophoblast transport function for a number of compounds. BeWo cells, besides exhibiting morphological properties, producing biochemical marker enzymes and secreting hormones characteristic of normal trophoblasts, rapidly form a confluent polarized monolayer, being particularly attractive for studies on transplacental kinetics (*19*). Indeed, they have been shown to exhibit polarized nutrient uptake systems (*21*, *22*) and polarized transcellular transport of transferrin (*23*) and serotonin (*24*).

MATERIALS AND METHODS

BeWo Cell Culture—The BeWo cell line was obtained from the American Type Culture Collection (ATCC CCL-98, Rockville, MD, USA) and was used between passage number 34 and 65. The cells were maintained in a humidified atmosphere of 5% CO_2-95% air, and were grown in Ham's F12K medium containing 2.5 g/l sodium bicarbonate, 10% heat-inactivated fetal calf serum and 1% antibiotic/antimycotic solution. Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:2, and sub-cultured in plastic culture dishes (21 cm^2; Ø 60 mm; TPP®, Trasadingen, Switzerland). For the transport studies, BeWo cells were seeded on collagen-coated 24-well plastic cell culture clusters (2 cm^2; Ø16 mm; TPP®), and were used after 3–5 days in culture (90–100% confluence). At this moment, each square centimetre contained about 60 µg cell protein.

Transport Studies—The transport experiments were performed in glucose-free HEPES-buffered solution with the following composition (in mM): 140 NaCl, 5 KCl, 20 HEPES-NaOH, 2.5 $MgSO_4$, 1 $CaCl_2$, pH 7.4. Initially, the culture medium was aspirated and the cells were washed with buffer at 37°C; then the cell monolayers were pre-incubated for 20 min in buffer at 37°C. Uptake was initiated by the addition of 0.3 ml buffer at 37°C containing 1 µM ^{3}H-2-deoxy-D-glucose (except in the experiments for determination of the kinetics of ^{3}H-DG uptake). Incubation was stopped after 6 min (except in the time-course experiments) by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml ice-cold buffer. The cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM Tris–HCl, pH 7.4), and placed at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Acute and Chronic Treatment of the Cells—The concentrations of compounds to test both acutely and chronically were chosen based on previous works from our group (*25–30*).

The acute effect of compounds on ^{3}H-DG uptake by BeWo cells was tested by pre-incubating (20 min) and incubating cells with ^{3}H-DG (1 µM; 6 min) in the presence of the compounds to be tested.

The chronic effect of compounds on ^{3}H-DG uptake by BeWo cells was tested by cultivating 3-day-old cell cultures (90–95% confluence) in culture medium containing the compounds to be tested. The medium was renewed daily, and the transport experiments were performed after 48 h. The transport experiments were identical to the experiments described above, except that there was no pre-incubation period, and cells were incubated with ^{3}H-DG for 6 min in the absence of drugs.

Assessment of Cell Viability—The effect of the dietary compounds on BeWo cell viability was determined by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (*31*).

To test whether the compounds that had an acute effect (26 min) upon ^{3}H-DG uptake affected cellular viability, BeWo cells were incubated for 3 h at 37°C in 500 µl of culture medium with 0.5 mg/ml MTT solution. In the last 26 min of this period, the compounds to be tested were added.

To test whether the compounds that had a chronic effect (48 h) upon ^{3}H-DG uptake affected cellular viability, BeWo cells where chronically treated with the compounds as described above. After 45 h of treatment, 50 µl MTT solution (5 mg/ml) was added to each well. The cells were then further incubated for 3 h at 37°C.

The MTT solution was removed after the 3 h incubation period, and the cells were lysed by addition of 200 µl DMSO followed by plate shaking for 10 min at room temperature. Optical density for the solutions in each well was determined at both 550 and 650 nm. Optical density at 650 nm corresponds to unspecific light absorption and was subtracted from the OD at 550 nm to give the OD value specific to formazan crystals derived from MTT cleavage.

Protein Determination—The protein content of cell monolayers was determined as described by Bradford (*32*), using human serum albumin as standard.

Calculations and Statistics—For the analysis of the time-course of ^{3}H-DG uptake, the parameters of Eq. 1 were fitted to the experimental data by a non-linear regression analysis, using a computer-assisted method (*33*).

$$A(t) = \frac{k_{in}}{k_{out}}\left(1 - e^{-k_{out}t}\right)$$

$A(t)$ represents the accumulation of ^{3}H-DG at time t, k_{in} and k_{out} the rate constants for inward and outward transport, respectively, and t the incubation time. A_{max} is defined as the accumulation at steady state ($t \to \infty$). K_{in} is given in picomoles milligrams protein^{-1} min^{-1} and k_{out} in min^{-1}. In order to obtain clearance values, k_{in} was converted to micro litre milligram protein^{-1} min^{-1}.

For the analysis of the saturation curve, the parameters of the Michaelis–Menten equation were fitted to the experimental data by using a non-linear regression analysis, using a computer-assisted method (*33*).

For calculation of IC_{50} values, the parameters of the Hill equation for multi-site inhibition were fitted to the

experimental data by a non-linear regression analysis, using a computer assisted method (*33*).

Arithmetic means are given with SEM and geometric means with 95% confidence intervals. n represents the number of replicates of at least two different experiments. Statistical significance of the difference between two groups was evaluated by the Student's t-test. Differences were considered to be significant when $P < 0.05$.

Materials—^{3}H-2-deoxy-D-glucose (deoxy-D-glucose, 2-[1,2-^{3}H]; specific activity 40–50 Ci/mmol) (Amersham Pharmacia Biotech, Buckinghamshire, UK); antibiotic/antimycotic solution (100 U ml^{-1} penicillin; 100 µg ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B), (+)catechin hydrate, chrysin, collagen type I, cytochalasin B (from *Diechslera dematioidea*), 2-deoxy-D-glucose, epicatechin, EGCG [(–) epigallocatechin-3-gallate], Ham's F12 K (nutrient mixture F12-Ham Kaighn's modification), HEPES (N-2-hydroxyethylpiperazine-N′2-ethanesulphonic acid), myricetin, MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide), (–)nicotine hydrogen tartrate, quercetin dihydrate, resveratrol, rutin, theophylline, trypsin-EDTA solution (Sigma, St Louis, MO, USA); DMSO (dimethylsulphoxide), Triton X-100 (Merck, Darmstadt, Germany); fetal calf serum (Invitrogen Corporation, Carlsbad, CA, USA); (±)-amphetamine, (±)-MDMA (ecstasy; 3,4-methylenedioxymetamphetamine), THC [(–)-Δ^9-tetrahydrocannabinol (tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol] (Cerilliant Corporation, Round Rock, TX, USA); acetaldehyde (May & Baker, Dagenham, UK); caffeine (BDH Laboratory Chemicals Ltd., Poole, UK); cocaine hydrochloride (Uquipa, Lisbon, Portugal).

Xanthohumol was kindly donated by Eng. José M. Machado Cruz, from iBeSa – Instituto de Bebidas e Saúde (S. Mamede Infesta, Portugal).

The drugs to be tested were dissolved in water, ethanol, methanol, DMSO or HCl 0.01 M, the final concentration of these solvents being 1% in the buffer or 0.1% in the culture media for acute or chronic treatments, respectively. Controls for these drugs were run in the presence of the solvent. None of these solvents significantly affected ^{3}H-DG uptake by BeWo cells (data not shown).

RESULTS

Time-Course of ^{3}H-DG Uptake—In a first series of experiments, we determined the time-course of ^{3}H-DG accumulation by BeWo cells. For this, cells were incubated with 1 µM of ^{3}H-DG for various periods of time, in the absence or presence of cytochalasin B.

As shown in Fig. 1, BeWo cells accumulated ^{3}H-DG in a time-dependent manner. Analysis of the time-course of ^{3}H-DG accumulation revealed a k_{in} of 24.1 ± 4.1 µl mg protein^{-1} min^{-1}, a k_{out} of 0.045 ± 0.010 min^{-1} and an A_{max} of 535.7 ± 46.8 pmol mg protein^{-1}. In other words, an amount of BeWo cells corresponding to 1 mg cell protein removed ^{3}H-DG present in 24.1 µl of buffer, and simultaneously 4.5% of intracellular ^{3}H-DG left the cells per minute. In the presence of cytochalasin B, the k_{in} was reduced to 8.9 ± 1.4 µl mg protein^{-1} min^{-1} and the A_{max} to 339.0 ± 35.7 pmol mg protein^{-1}. The k_{out}, however, was not significantly changed in the presence of this compound (Fig. 1).

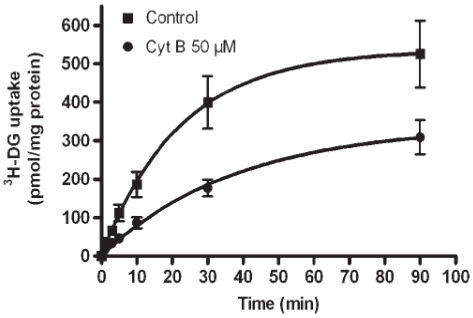

Fig. 1. **Time-course of ^{3}H-DG apical uptake by BeWo cells.** Cells were incubated at 37°C with 1 µM ^{3}H-DG for various periods of time, in the absence (control; $n = 6$) or presence of cytochalasin B 50 µM (Cyt B; $n = 5$–6). Shown are arithmetic means ± SEM.

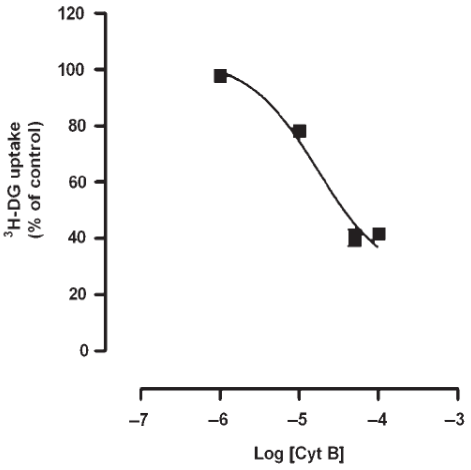

Fig. 2. **Effect of increasing concentrations of cytochalasin B upon ^{3}H-DG apical uptake by BeWo cells.** Cells were incubated at 37°C for 6 min, in the absence ($n = 14$) or presence of cytochalasin B ($n = 6$–9). Shown are arithmetic means ± SEM.

Analysis of the time-course of ^{3}H-DG uptake also showed that uptake was linear with time for up to 6 min of incubation, after which uptake reached a plateau (Fig. 1). On the basis of this information, a 6-min incubation time was selected as the standard incubation time in subsequent experiments.

Specificity of ^{3}H-DG Uptake—Next, we investigated the acute effect of increasing concentrations of cytochalasin B upon ^{3}H-DG uptake by BeWo cells (Fig. 2). This compound inhibited ^{3}H-DG uptake in a concentration-dependent manner, having a maximal inhibitory effect of 60%. The calculated IC_{50} of cytochalasin B in relation to ^{3}H-DG uptake by BeWo cells was 17.0 (8.9–32.6) µM ($n = 6$–9).

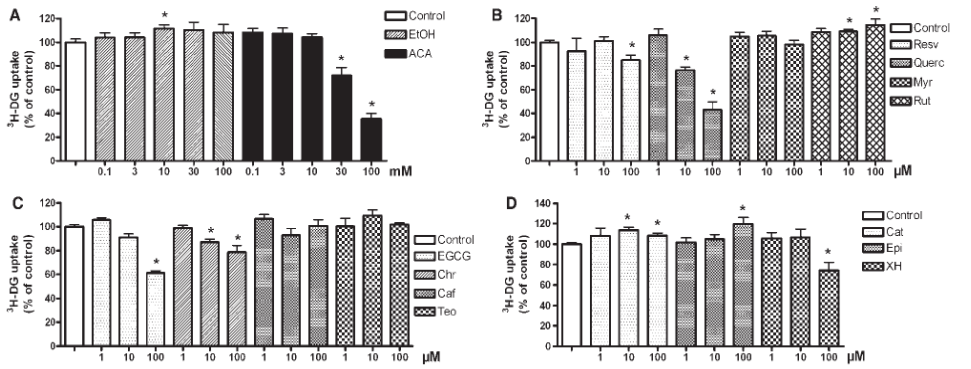

Fig. 3. **Effect of acute exposure of BeWo cells to different concentrations of (A) ethanol (EtOH; $n = 5$–6), acetaldehyde (ACA; $n = 3$–4), (B) resveratrol (Resv; $n = 9$), quercetin (Querc; $n = 6$), myricetin (Myr; $n = 6$), rutin (Rut; $n = 6$), (C) EGCG ($n = 6$), chrysin (Chr; $n = 5$), caffeine (Caf; $n = 5$–7), teophylline (Teo; $n = 4$) and (D) catechin (Cat; $n = 8$), epicatechin (Epi; $n = 5$), xanthohumol (XH; $n = 6$) on ^{3}H-DG apical uptake.** BeWo cells were incubated at 37°C with 1 μM ^{3}H-DG for 6 min, in the absence (control; $n = 8$–30) or presence of the compound. Shown are arithmetic means ± SEM. *Significantly different from control ($P < 0.05$).

We also investigated the acute effect of insulin upon ^{3}H-DG uptake by BeWo cells. Insulin (0.1, 1 or 10 μg/ml) did not affect ^{3}H-DG uptake (uptake in the presence of increasing concentrations of insulin corresponded to 100.8 ± 2.6, 97.0 ± 3.3 and 94.4 ± 3.1% of control, respectively; $n = 6$).

Effect of Dietary Bioactive Compounds on ^{3}H-DG Uptake—Acute Effect

The acute (26 min) effect of different concentrations of ethanol or acetaldehyde (0.1–100 mM) upon ^{3}H-DG uptake by BeWo cells was investigated (Fig. 3). With the exception of an 11% increase observed with 10 mM ethanol, this compound had no significant effect upon ^{3}H-DG uptake. On the other hand, acetaldehyde reduced ^{3}H-DG uptake in a concentration-dependent manner (producing a maximal reduction of uptake of 65%).

Next, the acute effect of increasing concentrations (1–10–100 μM) of several distinct polyphenols (catechin, chrysin, epicatechin, epigallocatechin-3-gallate, isoxanthohumol, myricetin, quercetin, resveratrol, rutin and xanthohumol) and two metilxanthines (caffeine and teophylline) was tested (Fig. 3). Quercetin and chrysin inhibited ^{3}H-DG uptake in a concentration-dependent manner, causing a maximal reduction in ^{3}H-DG uptake to 43 and 79% of control, respectively. Moreover, the highest concentration of resveratrol, EGCG and xanthohumol also caused a reduction in uptake (to 85, 61% and 74% of control, respectively). On the other hand, rutin, epicatechin and catechin caused an increase in the uptake of ^{3}H-DG (to a maximum of 114, 119 and 114% of control, respectively). Finally, myricetin and the two methylxanthines tested were devoid of effect (Fig. 3).

Chronic Effect

The chronic (48 h) effect of two different concentrations of ethanol and acetaldehyde (0.1 and 1 mM) on ^{3}H-DG uptake by BeWo cells was next investigated. None of these agents was able to change ^{3}H-DG uptake (data not shown). The effect of polyphenols and methylxanthines was also evaluated (Fig. 4). Myricitin and rutin increased ^{3}H-DG uptake in a concentration-dependent way, and teophylline decreased it in a concentration-dependent way. All the other compounds tested were devoid of effect (Fig. 4).

Effect of Drugs of Abuse on ^{3}H-DG Uptake—Acute Effect

The acute effect of increasing concentrations of some drugs of abuse [nicotine (1–100 μM), cocaine (0.1–10 μM), MDMA (0.1–10 μM), amphetamine (0.1–10 μM) and THC (0.01–1 μM)] on ^{3}H-DG uptake by BeWo cells has also been investigated. Surprisingly, none of the drugs tested had any significant effect upon ^{3}H-DG uptake (data not shown).

Chronic Effect

The chronic effect of different concentrations of these drugs of abuse was also investigated. We observed that both concentrations of amphetamine and MDMA, and the lowest concentration of THC, produced a small but significant reduction in ^{3}H-DG uptake (Fig. 4). Nicotine (0.1, 1 and 1 μM) and cocaine (0.25 and 2.5 μM) were devoid of effect (data not shown).

Effect of Dietary Bioactive Compounds and Drugs of Abuse on Cell Viability—In order to assess the cytotoxicity of the tested compounds, we determined both the acute and chronic effect of these compounds upon cell viability.

Acutely, none of the dietary bioactive compounds had any significant effect on cell viability, with the exception of chrysin and theophylline. Chrysin (10 and 100 μM; $n = 6$) reduced the viability to 79 ± 9 and 82 ± 6% of control, respectively. Theophyllin, on the other hand, increased the cellular viability (to 113 ± 1% of control; $n = 4$) at the lowest concentration tested (1 μM), but decreased it (to 91 ± 1% of control; $n = 4$) at the highest concentration used (100 μM). In relation to the drugs of abuse, all of them, with the exception of THC, reduced cellular viability.

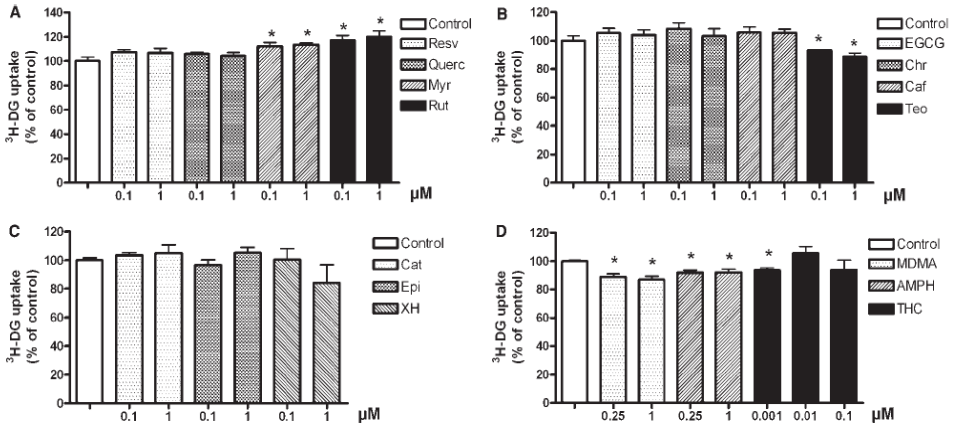

Fig. 4. **Effect of chronic exposure of BeWo cells to different concentrations of (A) resveratrol (Resv; $n = 4$), quercetin (Querc; $n = 4$), myricetin (Myr; $n = 4$), rutin (Rut; $n = 5$–6), (B) EGCG ($n = 6$), chrysin (Chr; $n = 4$), caffeine (Caf; $n = 4$), teophylline (Teo; $n = 4$), (C) catechin (Cat; $n = 5$), epicatechin (Epi; $n = 5$–8), xanthohumol (XH; $n = 6$), and (D) MDMA ($n = 6$), amphetamine (AMPH; $n = 6$), THC ($n = 6$–9) on ^{3}H-DG apical uptake.** BeWo cells were cultured for 48 h in the presence of different concentrations of the compound or the respective solvent (control; $n = 12$–15). For uptake experiments, cells were incubated at 37°C with 1 μM ^{3}H-DG for 6 min. Shown are arithmetic means ± SEM. *Significantly different from control ($P < 0.05$).

Cocaine decreased it in a concentration-dependent manner (0.1, 1 and 10 μM of this agent caused a reduction to 92 ± 2, 84 ± 5 and 81 ± 4% of control, respectively; $n = 12$). Moreover, nicotine and MDMA produced an about 12% reduction in cellular viability, but only at the lowest concentration tested (1 and 0.1 μM, respectively; $n = 8$–9). The higher concentrations tested (10 and 100 μM nicotine and 1 and 10 μM MDMA; $n = 8$–9) had no effect. Finally, 1 μM amphetamine ($n = 9$) reduced the viability in 6% (but 0.1 and 10 μM of this drug had no effect; $n = 8$–9).

Chronically, none of the dietary bioactive compounds tested affected the cellular viability, with the exception of xanthohumol, which at the highest concentration tested (1 μM) increased viability to 109 ± 3% of control ($n = 6$). Also, none of the drugs of abuse tested chronically had any significant effect upon BeWo cellular viability (data not shown).

Characterization of the Effect of Dietary Bioactive Compounds and Drugs of Abuse on ^{3}H-DG Uptake—Effect of the Compounds in the Presence of Cytochalasin B

In order to better characterize the inhibitory or stimulatory effect of the compounds upon ^{3}H-DG uptake, the effect of the compounds, which, acutely or chronically, affected ^{3}H-DG uptake, was assessed in the presence of 50 μM cytochalasin B. If the compounds are interfering with GLUT-mediated transport, they should not change the maximal inhibitory effect of cytochalasin B, which is a GLUT inhibitor, upon ^{3}H-DG uptake. On the other hand, if the compounds are interfering with non-GLUT-mediated glucose uptake, they would change the maximal inhibitory effect observed with cytochalasin B.

Acute Effect

Cytochalasin B (50 μM) reduced ^{3}H-DG uptake to about 50% of control (Fig. 5). When associated with cytochalasin B, none of the compounds which showed an acute effect upon ^{3}H-DG uptake (ethanol 10 mM, acetaldehyde 30 mM, resveratrol 100 μM, xanthohumol 100 μM, EGCG 100 μM, chrysin 100 μM, catechin 10 μM, epicatechin 100 μM and quercetin 10 μM) caused a significant change in the inhibitory effect of cytochalasin B, with the exception of acetaldehyde. In the presence of this compound, the uptake of ^{3}H-DG in the presence of cytochalasin B was further decreased by about 12% (Fig. 5).

Chronic Effect

Cytochalasin B (50 μM) for 48 h caused a small (83 ± 4% of control; $n = 9$) but significant decrease in ^{3}H-DG uptake by BeWo cells. None of the compounds which affected ^{3}H-DG uptake after a chronic exposure (myricietin 1 μM, rutin 1 μM, teophylline 1 μM, amphetamine 1 μM, MDMA 1 μM and THC 1 nM) were able to significantly change the inhibitory effect of cytochalasin B (data not shown).

Effect of Two Different Compounds in Combination—Acute Effect

As shown in Fig. 4, both 100 μM catechin and 100 μM epicatechin produced an increase in ^{3}H-DG uptake (to 108 ± 3 and 119 ± 6% of control, respectively). Unexpectedly, when these two drugs were combined, a reduction in ^{3}H-DG uptake (to 89 ± 3% of control; $n = 8$) was observed. On the other hand, the combination of epicatechin (100 μM) and xantho-humol (100 μM), which alone produced antagonic effects upon ^{3}H-DG uptake (epicatechin increased it to 119 ± 6% and xanthohumol decreased it to 74 ± 8% of control; see Fig. 3), resulted in a counterbalanced effect (95 ± 3% of control; $n = 6$).

Chronic Effect

The combined effect of myricetin (1 μM) and rutin (1 μM) was also investigated. When these two compounds were

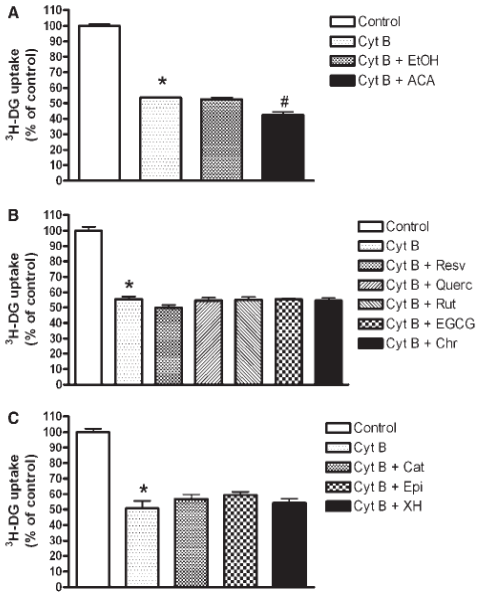

Fig. 5. **Effect of acute exposure of BeWo cells to different concentrations of (A) ethanol (EtOH; $n=6$), acetaldehyde (ACA; $n=6$), (B) resveratrol (Resv; $n=9$), quercetin (Querc; $n=9$), rutin (Rut; $n=9$), EGCG ($n=6$), chrysin (Chr; $n=9$), and (C) catechin (Cat; $n=6$), epicatechin (Epi; $n=8$), xantho-humol (XH; $n=8$) on ^{3}H-DG apical uptake in the presence of cytochalasin B 50 µM (Cyt B).** BeWo cells were incubated at 37°C with 1 µM ^{3}H-DG for 6 min, in the absence (control; $n=6$–12) or presence of the compound. Shown are arithmetic means ± SEM. *Significantly different from control ($P<0.05$). #Significantly different from cytochalasin B ($P<0.05$).

combined, the resultant effect (130 ± 5% of control; $n=9$) was higher than the effect of each of these drugs alone (114 ± 2 and 120 ± 5% of control for myricetin and rutin, respectively; see Fig. 4).

Effect of the Compounds Upon Kinetic Parameters of ^{3}H-DG Uptake—In this final set of experiments, we determined initial rates of ^{3}H-DG uptake (6-min incubation) at increasing substrate concentrations (1–10,000 µM). Uptake of ^{3}H-DG was found to be saturable with a K_m of 13.4 ± 0.5 mM and a V_{max} of 1210 ± 29 nmol mg protein^{-1} 6 min^{-1} ($n=4$).

In order to further characterize the acute inhibitory effect of quercetin, EGCG, acetaldehyde and xantho-humol upon ^{3}H-DG uptake, we examined the effect of these compounds (in the concentration causing the maximum inhibitory effect upon ^{3}H-DG uptake) on the kinetic parameters of ^{3}H-DG uptake by BeWo cells. The results (Table 1) show that all the compounds caused a significant decrease in the V_{max} of transport. Moreover, EGCG and acetaldehyde also significantly reduced the K_m value (*i.e.* increased the affinity of the transporter to ^{3}H-DG).

Table 1. **Acute effect of quercetin (QUERC 100 µM), EGCG (100 µM), acetaldehyde (ACA 30 mM) and xanthohumol (XH 100 µM) on the kinetic parameters of ^{3}H-DG uptake by BeWo cells.**

	K_m (mM)	V_{max} (nmol.mg protein^{-1} 6 min^{-1})	n
C (DMSO)	10.54 ± 0.31	976.8 ± 16.8	10
QUERC 100 µM	10.70 ± 0.56	475.3 ± 14.5*	6
EGCG 100 µM	9.16 ± 0.37*	772.5 ± 17.1*	6
C (H_2O)	13.44 ± 0.51	1210.0 ± 28.9	4
ACA 30 mM	6.63 ± 0.08*	500.7 ± 3.1*	4
C (EtOH)	12.23 ± 0.70	1330.0 ± 45.8	4
XH 100 µM	11.13 ± 0.55	1120.0 ± 32.8*	4

BeWo cells were incubated for 6 min, at 37°C, with increasing concentrations of ^{3}H-DG (1–10,000 µM), in the absence (control, C) or presence of the compounds. Shown are arithmetic means ± SEM. *Significantly different from control ($P<0.05$).

DISCUSSION

The aim of this work was to determine both the acute and chronic effect of some dietary bioactive compounds and drugs of abuse upon the apical uptake of glucose by BeWo cells. The BeWo cell line derives from a human gestational choriocarcinoma, and is a known cellular model of the human syncytiotrophoblast (*19*, *20*).

In our work, uptake of glucose was studied by using ^{3}H-DG as a substrate. DG is a D-glucose analogue that is transported efficiently by facilitated glucose transporters such as GLUT1 and GLUT2, but is poorly transported by SGLT1 (*34*). Moreover, once inside cells, this compound is phosphorylated by hexokinase to 2-deoxy-D-glucose-6-phosphate. This latter compound is metabolically inactive and is also poorly transportable across biological membranes. So, accumulation of ^{3}H-DG-6-phosphate in the cells is a good estimate of ^{3}H-DG rates of uptake.

We verified that ^{3}H-DG apical uptake by BeWo cells was time dependent, displayed saturable kinetics (V_{max} = 1210 ± 29 nmol mg protein^{-1} 6 min^{-1} and K_m = 13.4 ± 0.5 mM), and was significantly (by up to 60%) inhibited in the presence of cytochalasin B. Moreover, uptake of ^{3}H-DG was insulin-resistant. These results indicate that ^{3}H-DG apical uptake is mainly mediated *via* a facilitative glucose transport mechanism, most probably distinct from GLUT4. This conclusion is in perfect agreement with previous works showing that the BeWo choriocarcinoma cell line expresses GLUT1, GLUT3 and GLUT5 mRNA and protein (*10*, *35*, *36*), and with functional studies demonstrating that uptake of ^{3}H-DG by this cell line occurs through a facilitative glucose transport system (*36*, *37*). So, we may conclude that in our experiments, the apical uptake of ^{3}H-DG by BeWo cells was mainly mediated by a GLUT transport system, most probably GLUT1. The remainder of ^{3}H-DG uptake might correspond to non-GLUT-mediated transport, or, alternatively, to some non-specific adsorption of the compound to the cell membrane.

It is known that the increased glucose transport in malignant cells is associated with increased and deregulated expression of glucose transporter proteins, with over-expression of GLUT1 and/or GLUT3 being a characteristic feature. However, as stated in the INTRODUCTION section, the main transplacental transfer of glucose is

mediated by members of the GLUT family of transporters (*3–5*), predominantly the GLUT1 isoform (*6–10*). Moreover, similarly to human syncytiotrophoblasts, GLUT1 is the main glucose transporter expressed in BeWo cells (*36*). This indicates that the characteristics of glucose transport in normal trophoblast cells and BeWo cells are probably not very different.

One of the substances tested was ethanol. Ethanol is the most frequently used drug worldwide (chronic alcohol addiction affects at least 5% of the US population) (*38*), and its consumption is not uncommon during pregnancy. In the US, for example, between the years 2003 and 2004, 11% of pregnant women aged 15–44 years reported alcohol use, 4.5% reported binge drinking during the prior month and 0.5% reported heavy alcohol use during pregnancy (*39*, *40*). This agent is recognized as a potent teratogen in humans (*41*), and alcohol abuse during pregnancy can give rise to alcohol-related birth defects such as spontaneous abortion, decreased immune function, attention problems, hearing impairment (*42*), permanent fetal brain damage and a wide variety of manifestations, known as fetal alcohol spectrum disorder (FASD) (*43*). Among children with FASD, a small population present a specific set of anomalies [specific facial abnormalities, intrauterine growth retardation and significant impairments in neurodevelopment (*41*, *44–46*)]. One of the factors involved in the toxic effect of ethanol is the formation of its metabolite acetaldehyde at the maternal, placental and fetal level (*47*, *48*).

In our experiments, both ethanol and acetaldehyde affected the apical uptake of ^{3}H-DG only when tested acutely. However, their effects were very distinct: ethanol (10 mM) produced only a slight (10%) increase in ^{3}H-DG uptake whereas acetaldehyde decreased uptake in a concentration-dependent manner, to a maximum of 65% (with 100 mM). The effects of both compounds were not related to changes in the cellular viability. Moreover, from the experiments where the compounds were associated with cytochalasin B, we concluded that ethanol interacts with GLUT, whereas acetaldehyde exerts its effect, at least partially, by a mechanism distinct from interaction with GLUT. Finally, acetaldehyde affected the kinetic parameters of ^{3}H-DG uptake, decreasing both the K_m and the V_{max}.

Among the various possible mechanisms underlying FASD, there is some evidence that ethanol toxicity during pregnancy is associated with altered placental transport function for some nutrients (glucose, aminoacids and folic acid) (*23*, *47*, *49–52*). However, in our experiments, ethanol was devoid of significant effect upon the apical uptake of ^{3}H-DG, and so they do not confirm the involvement of placental glucose uptake reduction in the deleterious effects of ethanol during pregnancy. Interestingly enough, inhibition of placental glucose transport was observed in rats fed ethanol throughout pregnancy (*51*, *52*). We may speculate that differences between these works and the present one relate to the duration of placental exposure to this agent. On the other hand, we may speculate that inhibition of the placental uptake of glucose may be involved in the negative effects of acetaldehyde. Interestingly enough, 20 mM of this agent also reduced the placental transport of aminoacids (*53*).

To further investigate the nutritional modulation of glucose placental uptake, we also investigated the effect of acute and chronic exposure of BeWo cells to different polyphenols (catechin, chrysin, epicatechin, epigallocatechin-3-gallate, myricetin, quercetin, resveratrol, rutin and xanthohumol) and methylxanthines (caffeine and theophylline) present in alcoholic (*e.g.* red wine) and non-alcoholic (*e.g.* green and black tea) drinks. The increasing interest in studying polyphenols arises from their antioxidant properties and their potential role in the prevention of cancer and cardiovascular, neurodegenerative and inflammatory diseases (*54–56*). Polyphenols and methylxanthines freely cross the placenta (*57*, *58*) and thus their effect on the placenta and fetus should be investigated. In relation to caffeine and theophylline, the effect of these substances was investigated because drinks containing these substances (*e.g.* coffee and black tea) are often consumed by pregnant women. For instance, 75% of US pregnant women consume low or moderate amounts of caffeine (*59*). Although there is still some controversy concerning the risks associated with the maternal use of caffeine during pregnancy (*e.g.* spontaneous abortion and intrauterine growth restriction), the FDA recommends that pregnant women should avoid the ingestion of caffeine (*59*, *60*).

Acutely, ^{3}H-DG apical uptake was significantly reduced by resveratrol, xanthohumol and EGCG (100 μM), and by quercetin and chrysin (10 and 100 μM). On the other hand, rutin and catechin (10 and 100 μM) and epicatechin (100 μM) increased ^{3}H-DG apical uptake. Together, catechin and epicatechin (both at 100 μM) decreased the apical uptake of ^{3}H-DG, whereas epicatechin and xanthohumol (both at 100 μM) counterbalanced each one's isolated effect. From the experiments in the presence of cytochalasin B, all these compounds seem to interact with GLUT in order to exert their effects. From the analysis of the effect of these compounds on the kinetic parameters of ^{3}H-DG uptake, quercetin and xanthohumol seem to be non-competitive inhibitors of ^{3}H-DG uptake, whereas epigallocatechin-3-gallate decreased both the K_m and V_{max} values.

Chronically, rutin and myricetin (0.1 and 1 μM) increased the apical uptake of ^{3}H-DG. When associated, uptake was further increased. On the contrary, theophylline (0.1 and 1 μM) decreased ^{3}H-DG uptake. All these compounds seem to exert their effect by interacting with GLUT.

The atypical behaviour of acetaldehyde and EGCG (decreasing both the K_m and V_{max} of ^{3}H-DG uptake) may be explained by binding of these compounds to an allosteric site of GLUT, inducing an alteration in the conformation of the active site, thus increasing the affinity for the substrate and decreasing the transporter's capacity for high concentrations of the substrate.

To our knowledge, the effect of polyphenolic compounds upon the placental uptake of ^{3}H-DG have not been studied. In agreement with our results, some of these compounds, when tested acutely, were also found to inhibit (resveratrol, quercetin and EGCG) or stimulate (catechin, epicatequin) GLUT-mediated glucose uptake in other cell types (*61–65*), but other compounds (myricetin and rutin) had a distinct effect from that observed by us (*61*, *62*). However, the chronic effect of these compounds was not evaluated before. The daily intake of polyphenols

in the US population (1 g/day on average) originates blood concentrations in the range 1–10 μM (*66*), but dietary supplementation with polyphenols might originate higher blood levels of these compounds.

It was recently shown that GLUTs are upregulated by hypoxia, *via* a hypoxia-inducible factor-1 (HIF-1)-mediated mechanism, in trophoblast cells (*67*). Interestingly enough, some polyphenolic compounds have been shown to modulate HIF-1 activity (*68–69*). So, it is possible that the effect of polyphenols on ^{3}H-DG uptake in BeWo cells is HIF-1 mediated.

Concerning the effect of methylxanthines, although in our experiments ^{3}H-DG uptake was reduced by chronic theophylline only, some previous studies have shown a negative effect of both caffeine and theophylline upon GLUT-mediated transport of glucose (*70–72*). However, it is important to note that in these studies only the acute effect of high (mM) concentrations of methylxanthines was assessed.

Finally, we also determined the effect of the drugs of abuse amphetamine, MDMA, THC, nicotine and cocaine upon the apical uptake of ^{3}H-DG by BeWo cells. The consumption of drugs of abuse has been increasing among young women; ethanol and tobacco, consumed by about half of the US pregnant women, being the most prevalent ones (*73*). Moreover, a study conducted in the late 90s reported that 5–6% of the US pregnant women consumed illicit drugs of abuse, with marijuana and cocaine being the most consumed ones (*73*, *74*). It is known that tobacco (and its ingredient nicotine) may cause spontaneous abortion, premature delivery, intrauterine growth restriction and low birth weight (*75*). As to illicit drugs, some studies have show that there is an association between their use during pregnancy and adverse effects on both maternal (*e.g.* spontaneous abortion and premature delivery) and fetal (*e.g.* intrauterine growth restriction, congenital malformations) health (*73*, *76*).

When tested acutely, none of the drugs of abuse significantly changed apical ^{3}H-DG uptake. However, chronic exposure of the cells to MDMA, amphetamine (0.25 and 1 μM) or THC (1 nM) caused a small (±10%) but significant reduction in this parameter. The inhibitory effect of these drugs did not result from a cytotoxic effect. Moreover, these drugs seem to interact with GLUT in order to exert their effects. The blood concentration of amphetamine in therapeutic and drug users [0.3–0.8 and 1–2 μM, respectively; (*77*)] is on the same order of magnitude of the concentrations found to affect ^{3}H-DG uptake, in this study.

Interestingly enough, an inhibitory effect of some of these drugs of abuse in the placental transport of other nutrients (aminoacids and L-carnitine) and monoamines (dopamine, noradrenaline and serotonin) has been previously described (*77–79*).

In summary, our results show a detrimental effect of acute acetaldehyde, resveratrol, xanthohumol, EGCG, quercetin and chrysin and of chronic theophylline, THC, MDMA and amphetamine and a benefic effect of acute rutin, catechin and epicatechin and of chronic rutin and myricetin on ^{3}H-DG uptake by BeWo cells. In relation to the acute effect, quercetin and xanthohumol seem to be non-competitive inhibitors of ^{3}H-DG uptake, whereas both EGCG and acetaldehyde decreased both the K_m and V_{max} values. Apart from acetaldehyde, all these compounds seem to interact with GLUT in order to exert their effects. Moreover, apart from the acute inhibitory effect of chrysin, both the acute and chronic effects of these compounds on ^{3}H-DG uptake do not result from cytotoxic effects on cells.

Finally, it is worth noticing the fact that acute and chronic treatment with all the dietary bioactive compounds did not produce parallel results. Therefore, care should be taken when speculating about chronic effects from acute effects, and *vice versa*. Moreover, our results also show that the effect of polyphenolic compounds in combination may be very different from the expected ones taking into account the effect of each of these compounds alone. So, care should be taken when speculating for the effect of a drink based on the effect of one component only.

This work was supported by Fundação para a Ciência e a Tecnologia and Programa Ciência, Tecnologia e Inovação do Quadro Comunitário de Apoio [POCTI/SAU-FCF/59382/2004].

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European Journal of Pharmacology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Oxidative stress induced by *tert*-butylhydroperoxide interferes with the placental transport of glucose: *in vitro* studies with BeWo cells

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ARTICLE INFO

Article history:
Received 22 February 2013
Received in revised form
10 October 2013
Accepted 16 October 2013

Keywords:
Glucose
Oxidative stress
Placenta
Transport

ABSTRACT

Increased oxidative stress is implicated in the onset and progression of prevalent pregnancy disorders (*e.g.* gestational diabetes and fetal growth restriction), and in programming the fetus to develop metabolic diseases later in life. Since the molecular mechanisms underlying these effects of oxidative stress are largely unexplored, we aimed to investigate if the placental transport of glucose – the main energetic substrate for the fetus and placenta – is altered by oxidative stress. In a human syncytiotrophoblast (STB) cell model, the BeWo cell line, oxidative stress was induced by treatment with 100 μM *tert*-butylhydroperoxide (*tert*-BOOH) for 24 h. *Tert*-BOOH decreased the steady-state intracellular accumulation (A_{max}) of [^{3}H]2-deoxyglucose ([^{3}H]DG) mediated by both facilitative (GLUT) and non-facilitative (non-GLUT) glucose transporters. These effects were not associated with a change in the mRNA expression level of GLUT1, the major placental glucose transporter. Also, they seemed to be independent from phosphoinositide 3-kinase and protein kinase C signaling pathways and were unchanged either by inhibitors of free radical-generating enzymes or by free radical scavengers. In contrast, the dietary polyphenols quercetin, epigallocatechin-3-gallate and resveratrol completely reversed the inhibitory effect of *tert*-BOOH upon [^{3}H]DG accumulation through a specific effect on GLUT-mediated transport. Finally, *tert*-BOOH induced an increase in the transepithelial permeability to [^{3}H]DG in the apical-to-basal direction, apparently related to an increase in its paracellular transport. In conclusion, *tert*-BOOH-induced oxidative stress reduces STB accumulation of glucose associated with an increase in its transepithelial permeability. This effect may contribute to the deleterious consequences of pregnancy disorders associated with oxidative stress.

1. Introduction

One of the major functions of the placenta is to mediate the transport of nutrients from the mother to the fetus over the course of gestation. This function depends on the activity of specific transporters present at the apical (maternal-facing) and basal (fetal-facing) membranes of the syncytiotrophoblast (STB) epithelium (Jansson et al., 2009).

Glucose is the primary substrate for energy metabolism in the feto-placental unit, and together with amino acids, it constitutes the primary stimuli for fetal secretion of the growth-promoting hormone insulin (Jansson et al., 2009). Since fetal glucose production is minimal (Magnusson et al., 2004), placental transport constitutes the primary source of glucose to the fetus (Baumann et al., 2002; Carter, 2012). Accordingly, alterations in glucose transport and metabolism at the STB level are strongly associated with aberrant fetal growth (Baumann et al., 2002; Desoye et al., 2011; Illsley, 2000; Magnusson et al., 2004), which increases the risk of perinatal complications and predispose the newborn to develop cardiovascular and metabolic diseases later in life (Jansson et al., 2009; Vo and Hardy, 2012).

Placental transport of glucose occurs mainly through facilitative glucose transporters (GLUT). At least five different GLUT isoforms are expressed in the human STB: GLUT1, 3, 4, 9 and 12. However, the primary isoform responsible for glucose transport across the apical and basal membranes of the STB in term pregnancy is GLUT1 (Baumann et al., 2002; Carter, 2012; Jansson et al., 2009). GLUT1 distribution in the STB is asymmetric, with a greater expression and activity in the apical membrane, which assures that glucose is transported down its concentration gradient from maternal to fetal circulation (Baumann et al., 2002; Carter, 2012).

An increasing amount of evidence implicates oxidative stress in the pathophysiology of prevalent pregnancy complications such as miscarriage (Myatt and Cui, 2004), preeclampsia (Siddiqui et al., 2010), fetal growth restriction (Son et al., 2004; Takagi et al., 2004) and gestational diabetes (Coughlan et al., 2004; Lappas et al., 2011; Peuchant et al., 2004). Moreover, increased oxidative stress at the

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http://dx.doi.org/10.1016/j.ejphar.2013.10.023

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intrauterine environment has been demonstrated to program the fetus to develop metabolic and cardiovascular complications later in life (Dong et al., 2013; Giussani et al., 2012). Given that there are major gaps both in the understanding of the cellular and molecular mechanisms that underlie the pathological and programming effects of oxidative stress during pregnancy, and in the study of the impact of oxidative stress upon placental and fetal nutrition, we aimed to investigate the effect of oxidative stress upon the placental uptake of glucose, by using a cell model of human STB, the BeWo cell line. This cell line is a well-characterized and widely used cell model to investigate placental glucose transport, since GLUT expression (Baumann et al., 2007; Shah et al., 1999) and activity (Illsley, 2000; Vardhana and Illsley, 2002) (particularly of GLUT1) in BeWo cells are similar to that of primary cultured trophoblast cells (Baumann et al., 2007) and human term placentae (Baumann et al., 2002).

2. Materials and methods

2.1. Materials

2-[1,2-^{3}H(N)]deoxy-D-glucose – specific activity 60 mCi/mmol, and D-[^{14}C(U)]sorbitol – specific activity 300 mCi/mmol (American Radiolabeled Chemicals, St. Louis, MO, USA); albumin from bovine serum, chelerythrine chloride, collagen type I, cytochalasin B (from *Diechslera dematioidea*), decane, 5,5′-dithiobis(nitrobenzoic) acid, 2,4-dinitrophenylhydrazine, (-)-cis-3,3′,4′,5,5′,7-hexahydroxy-fla-vane-3-gallate ((−) epigallocatechin-3-gallate), fetal calf serum, glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*), Ham's F12K medium (Kaighn's modification), 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one (LY-294002) hydrochloride, *N*-acetyl-L-cysteine, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate, 3,3′,4′,5,6-pentahydroxy-flavone (quercetin) dihydrate, β-(4-hydroxyphenyl)-2,4,6-trihydroxypropiophenone, 2′,4′,6′-trihydroxy-3-(4-hydroxyphenyl)propiophenone, 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone (phloretin), 3,4′,5-trihydroxy-trans-stilbene (resveratrol), sodium pyruvate, phenol red sodium salt, sulforhodamine B, *tert*-butyl hydroperoxide (*tert*-BOOH), 2-thiobarbituric acid, trichloroacetic acid sodium salt, and 2-vinylpyridine (Sigma, St. Louis, MO, USA); and dimethylsulfoxide, D(+)-glucose and Triton X-100 (Merck, Darmstadt, Germany).

Compounds to be tested were dissolved in decane, water, or dimethylsulfoxide. The final concentration of these solvents in the buffer and culture medium was 1% (v/v).

2.2. BeWo cell culture

The BeWo cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ GmbH, ACC-458). Cells were used between passage numbers 16 and 49 and cultured as previously described (Araújo et al., 2008). For cell viability and cytotoxicity assays and for [^{3}H]2-deoxyglucose ([^{3}H]DG) apical uptake studies, cells were seeded on collagen-coated 24-well plastic cell culture clusters (2 cm^{2}; ∅16 mm; TPP, Trasadingen, Switzerland), and for measurement of glutathione levels, lipid peroxidation products and protein carbonyl groups, cells were seeded on collagen-coated 12-well plastic cells culture clusters (4 cm^{2}; ∅21 mm; TPP). Experiments were performed 6–7 days after the initial seeding (90–100% confluence). For transepithelial transport experiments, BeWo cells were seeded on collagen-coated Transwell® inserts (0.4 μm pore size, 12 mm diameter; Corning Costar, NY, USA) and experiments were performed 9–10 days after the initial seeding (100% confluence).

For cellular viability and cytotoxicity assays and quantification of oxidative stress biomarkers, confluent BeWo cells were exposed for 24 h to 1, 3, 10, 30, 100, 300 or 1000 μM of the oxidizing and free radical generating agent *tert*-BOOH (Tormos et al., 2004), or its solvent (decane), in fetal calf serum-free culture media.

2.3. Cellular viability and cytotoxicity assays

Cellular viability and cytotoxicity were quantified by measuring the activity of extracellular lactate dehydrogenase (LDH) and by the sulforhodamine B assay, respectively, as described elsewhere (Gonçalves et al., 2013).

2.4. Evaluation of tert-BOOH-induced oxidative stress in BeWo cells

Tert-BOOH has been previously shown by our group to induce oxidative stress in non-placental cell lines (Araújo et al., 2013; Couto et al., 2012; Gonçalves et al., 2013). To confirm that *tert*-BOOH induced oxidative stress in BeWo cells we measured total glutathione (GSX), GSSG and GSH levels, and the generation of lipid peroxidation products and protein carbonyl groups, as described previously (Gonçalves et al., 2013).

2.5. Transport studies

2.5.1. Apical uptake

The transport experiments were performed in glucose-free buffer solution containing (mM): 140 NaCl, 5 KCl, 20 HEPES–NaOH, 2.5 $MgSO_4$ and 1 $CaCl_2$, pH 7.4.

After treatment with *tert*-BOOH (100 μM; 24 h) or its solvent, BeWo cells were washed with buffer at 37 °C, and preincubated for 20 min. Incubation was then initiated by the addition of buffer containing 50 nM [^{3}H]DG for various periods of time. [^{3}H]DG is a D-glucose analog efficiently transported by GLUT transporters but poorly transported by sodium-dependent glucose transporters (Shah et al., 1999). Incubation was stopped by rinsing the cells with ice-cold buffer, and then cells were solubilized with 0.1% (v/v) Triton X-100 (5 mM Tris–HCl, pH 7.4). Radioactivity in the cells was measured by liquid scintillation counting and normalized for total cell protein, which was determined as described by Bradford (1976).

Tert-BOOH 100 μM and compounds to be tested (inhibitors of intracellular signaling pathways and antioxidants) were present during both the preincubation and incubation periods. Controls were run in the presence of the respective solvents, which did not significantly affect [^{3}H]DG uptake (results not shown).

GLUT-mediated transport was measured as the Na^{+}-independent and cytochalasin B (50 μM)-sensitive component of [^{3}H]DG accumulation and non-GLUT-mediated transport was calculated as the Na^{+}-independent and cytochalasin B (50 μM)-insensitive component of [^{3}H]DG accumulation. To measure Na^{+}-independent transport, cells were incubated in buffer in which 125 mM NaCl was isotonically replaced by LiCl. Cytochalasin B, a well-established inhibitor of GLUT isoforms (Araújo et al., 2008; Lappas et al., 2012; Shah et al., 1999), did not affect cellular viability at the tested concentration (50 μM) (results not shown).

2.5.2. Apical-to-basal transepithelial transport

Transepithelial [^{3}H]DG transport was determined by measuring the apical-to-basal passage of [^{3}H]DG across BeWo cell monolayers grown in Transwells®. Both apical-to-basal transport and intracellular accumulation of [^{3}H]DG were measured by adding buffer at 37 °C containing 20 nM [^{3}H]DG to the apical reservoir, after a preincubation period of 20 min. Transport was followed as a function of time, as samples (50 μl) were taken from the basal side every 30 min for 120 min and were replaced with equal volumes of buffer.

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Results were expressed as apparent permeability coefficient (P_{app}). *Tert*-BOOH was present in both apical and basal reservoirs throughout the experiment.

The effect of *tert*-BOOH upon paracellular transport across BeWo cells was assessed by (a) adding the paracellular marker phenol red (100 μM) to the apical reservoir at the beginning of the experiment and measuring (spectrophotometrically at 560 nm) the apical-to-basal transport after 120 min, and by (b) measuring the apical-to-basal transport of the paracellular marker [^{14}C] sorbitol (500 nM), as described above.

BeWo cell monolayer integrity and confluence was evaluated at the beginning and at the end of the experiment by measurement of transepithelial electrical resistance, using an epithelial voltohmmeter fitted with planar electrodes (EVOM; World Precision Instruments, Stevenage, UK). Measurements of transepithelial electrical resistance showed that BeWo cells formed a confluent monolayer from the beginning until the end of the permeability study both in the absence or presence of *tert*-BOOH (results not shown).

2.6. RNA extraction and real-time RT-PCR

RNA extraction, cDNA synthesis and real-time RT-PCR performed in BeWo cells are described in Supplementary data.

2.7. Quantification of lactate levels

Glucose metabolism was assessed by quantification of lactate in the extracellular medium after treatment of BeWo cells with 100 μM *tert*-BOOH for 24 h. Lactate concentration was measured by the lactate oxidase/peroxidase colorimetric assay, following manufacturer's instructions (Olympus Life and Material Science Europa GmbH, Hamburg, Germany). Lactate production was expressed in nmol mg prot^{-1}.

2.8. Calculations and statistics

The analysis of time-course of [^{3}H]DG apical uptake was performed by using a non-linear regression analysis, as previously described by our group (Araújo et al., 2008).

P_{app} values were determined over a 120-min flux period, as described by Santos et al. (2008).

Arithmetic means are given with S.E.M. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance followed by the Student–Newman–Keuls post-test. For comparison between two groups, Student's *t*-test was used. Differences were considered to be significant when $P < 0.05$.

The value of *n* indicates the number of replicates of at least two different experiments.

3. Results

3.1. Cytotoxicity of tert-BOOH and effect upon BeWo cell viability

In a first series of experiments, we evaluated the cytotoxic effect and the effect upon cell viability of exposure of BeWo cells to increasing concentrations of *tert*-BOOH (1–1000 μM) for 24 h. Exposure of cells up to 100 μM *tert*-BOOH did not affect cell viability (assessed with the extracellular lactate dehydrogenase assay) (Fig. 1A) and caused no cytotoxicity (assessed with the sulforhodamine B assay) (Fig. 1B). In contrast, higher concentrations (300–1000 μM) of *tert*-BOOH significantly decreased cell viability and induced cytotoxicity (Fig. 1A and B).

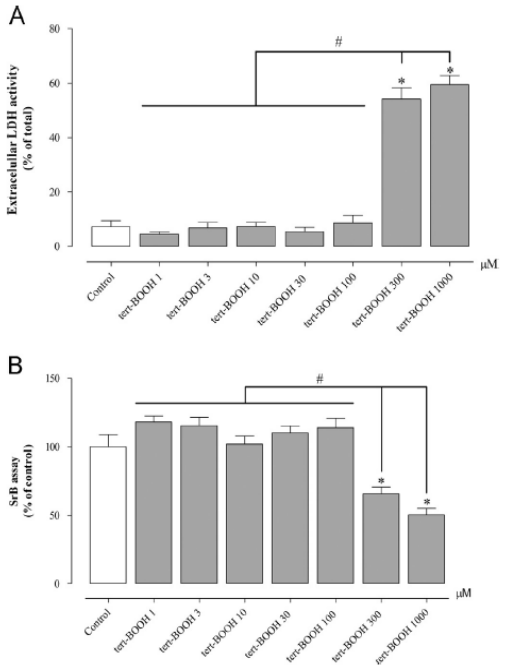

Fig. 1. Effect upon cell viability (a) and cytotoxicity (b) of *tert*-BOOH in BeWo cells. These parameters were determined after a 24 h-exposure of BeWo cells to 1–1000 μM *tert*-BOOH or to its solvent (control) (n=9–12). Shown are arithmetic means+S.E.M. *Significantly different from control ($P < 0.05$) and $^{\#}$significantly different from *tert*-BOOH 1–100 μM ($P < 0.05$).

3.2. Tert-BOOH efficiently induces oxidative stress in BeWo cells

Treatment of BeWo cells with 100 μM *tert*-BOOH for 24 h increased cellular GSX and GSSG levels. GSH levels did not change, probably due to its greater intracellular pool size, in comparison with GSSG, as high and similar GSH/GSSG ratios were found in control and *tert*-BOOH-treated cells (8.6 ± 1.6 and 9.1 ± 2.4, respectively; n=9) (Fig. 2).

Under the same condition, *tert*-BOOH induced an increase in the levels of the lipid peroxidation product malonaldehyde, an indicator of oxidative damage to lipids (Fig. 3A), and of protein carbonyl groups, an indicator of oxidative damage to proteins (Fig. 3B).

Since exposure of BeWo cells to 100 μM *tert*-BOOH for 24 h induced an increase in oxidative stress biomarkers while maintaining cellular viability and not causing cytotoxicity, this condition was used in subsequent experiments aimed at determining the effect of oxidative stress upon glucose transport in BeWo cells.

3.3. Tert-BOOH decreases the steady-state accumulation of [^{3}H]DG in BeWo cells

In a first series of experiments, we determined the effect of *tert*-BOOH upon [^{3}H]DG apical uptake over time (Fig. 4). *Tert*-BOOH caused a significant decrease in [^{3}H]DG intracellular accumulation at steady-state (A_{max}), as a result of a 2.6× increase in the rate constant of [^{3}H]DG outward transport (k_{out}) and of a not so marked (1.7×) increase in the rate constant of [^{3}H]DG inward transport (k_{in}) (Fig. 4A). Moreover, we verified that *tert*-BOOH decreased the A_{max} of both GLUT- and non-GLUT mediated uptake

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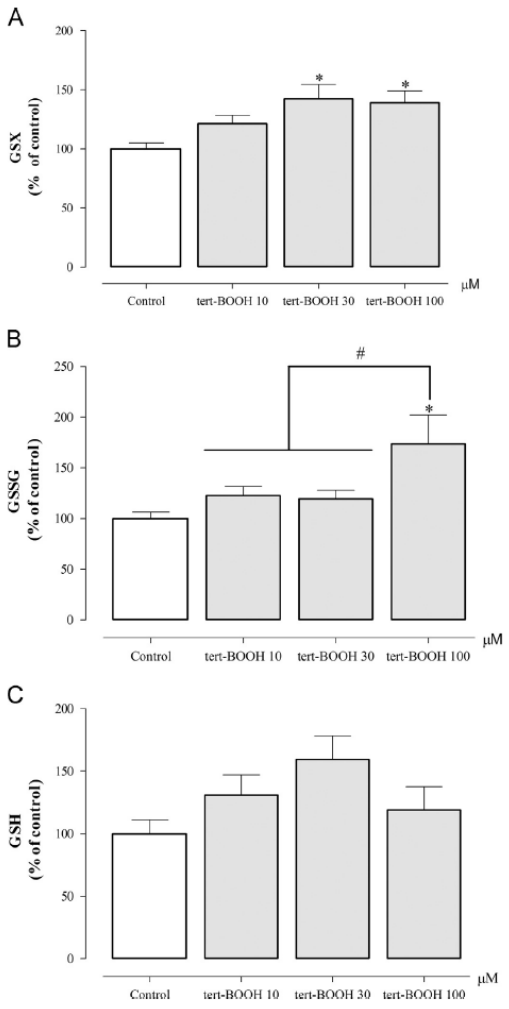

Fig. 2. Effect of *tert*-BOOH upon (a) total glutathione (GSx), (b) GSSG and (c) GSH levels in BeWo cells. These parameters were determined after a 24 h-exposure of BeWo cells to 10–100 µM *tert*-BOOH or to its solvent (control) ($n = 9$–16). Shown are arithmetic means + S.E.M. *Significantly different from control ($P < 0.05$) and #significantly different from *tert*-BOOH 10 and 30 µM ($P < 0.05$).

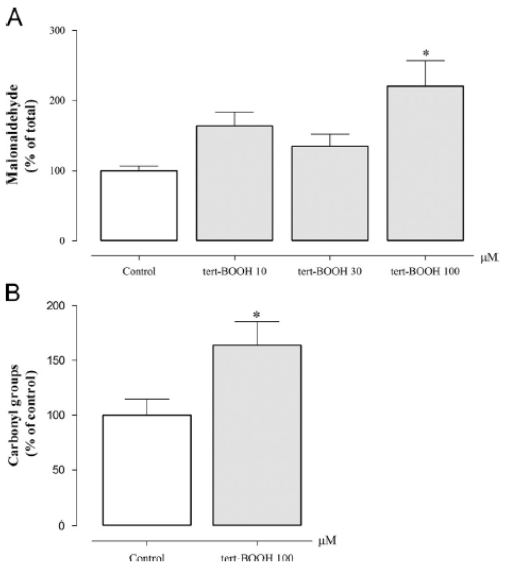

Fig. 3. Effect of *tert*-BOOH upon (a) malonaldehyde (a lipid peroxidation product) and (b) protein carbonyl levels in BeWo cells. These parameters were determined after a 24 h-exposure of BeWo cells to 10–100 µM *tert*-BOOH or to its solvent (control) ($n = 15$–30). Shown are arithmetic means + S.E.M. *Significantly different from control ($P < 0.05$).

(from 8.8 ± 0.7 to 6.4 ± 0.4 pmol mg prot^{-1} and from 7.4 ± 0.8 to 4.5 ± 0.4 pmol mg prot^{-1}, respectively) (Fig. 4B).

GLUT1 is considered the main functional glucose transporter expressed in the STB (Baumann et al., 2002) and BeWo cells (Shah et al., 1999). By real-time RT-PCR, we verified that *tert*-BOOH did not affect the mRNA expression level of GLUT1 (results not shown).

3.4. PI3K and PKC inhibition does not alter the inhibitory effect of *tert*-BOOH upon [^{3}H]DG accumulation by BeWo cells

The involvement of phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC) on the effect of *tert*-BOOH upon [^{3}H]DG accumulation was next investigated. Both pathways have been shown to be important regulators of glucose transport in placental (Lappas et al., 2012; Riley et al., 2005) and non-placental cells (Fernandes et al., 2011; Han et al., 2006; Wieman et al., 2007), and their activity and expression have been shown to be modulated by oxidative stress (Li et al., 2011; Perez et al., 2006; Poli et al., 2004).

GLUT-dependent [^{3}H]DG accumulation was reduced (by 15%) in the presence of a PI3K specific inhibitor (LY-294002 1 µM) (Vlahos et al., 1994), suggesting that PI3K activation is required for GLUT-mediated uptake of [^{3}H]DG. On the other hand, non-GLUT-dependent accumulation was not affected by this compound (Fig. 5). As to PKC, neither total, nor GLUT or non-GLUT components of [^{3}H]DG accumulation were affected by the PKC specific inhibitor chelerythrine (0.1 µM) (Herbert et al., 1990) (Fig. 5).

The inhibitory effect of *tert*-BOOH upon total, GLUT and non-GLUT-mediated [^{3}H]DG accumulation was not modified by either LY-294002 or chelerythrine, excluding the involvement of PI3K and PKC in this effect (Fig. 5).

3.5. Polyphenols reverse the inhibitory effect of tert-BOOH upon [^{3}H] DG accumulation by BeWo cells

We also investigated the ability of some antioxidants (inhibitors of reactive oxygen species-generating enzymes, free radical scavengers and dietary polyphenols) to reverse the inhibitory effect of *tert*-BOOH upon [^{3}H]DG accumulation (Fig. 6).

The NADPH oxidase inhibitor apocynin (1 mM) (Johnson et al., 2002), the xanthine oxidase inhibitor allopurinol (1 mM) (Pacher et al., 2006), and the free radical scavengers *N*-acetyl-cysteine (0.1 mM) (Gallo et al., 2010) and α-tocopherol (1 mM) (Al-Gubory et al., 2010) were all devoid of the effect upon total (Fig. 6A), GLUT- and non-GLUT-dependent [^{3}H]DG accumulation (results not shown). Also, these compounds did not interfere with the effect of *tert*-BOOH upon total (Fig. 6A), GLUT- and non-GLUT-dependent

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[^{3}H]DG accumulation (results not shown). Thus, free radical production neither appears to affect [^{3}H]DG accumulation nor to be involved in the inhibitory effect of *tert*-BOOH upon it.

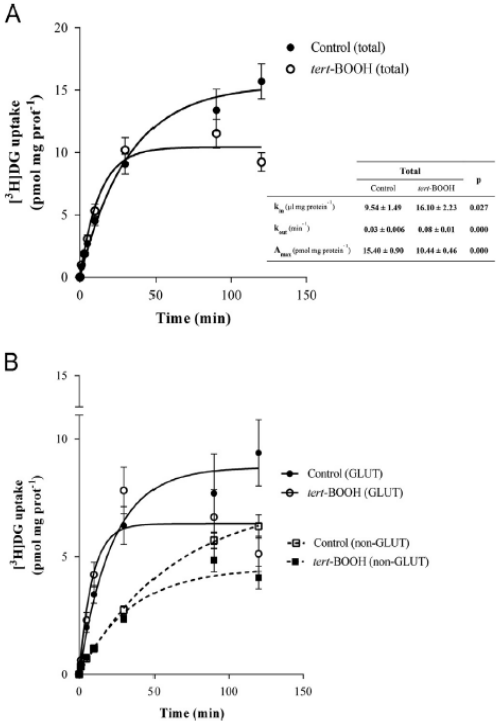

Fig. 4. Time-course of [^{3}H]deoxyglucose ([^{3}H]DG) uptake by BeWo cells after treatment with *tert*-BOOH (24 h; 100 μM). Cells were incubated at 37 °C for different periods of time with 50 nM [^{3}H]DG, pH 7.4, in buffer containing Na^+ (a) or in Na^+-free buffer in the absence or presence of cytochalasin B 50 μM (b) (n = 10–19). Analysis of the time courses allowed determination of the steady-state accumulation (A_{max}) and the rate constant for inward (k_{in}) and outward (k_{out}) transport. Shown are arithmetic means ± S.E.M.

Previous works from our group showed that the polyphenols epigallocatechin-3-gallate, quercetin and resveratrol were able to prevent the inhibitory effect of *tert*-BOOH upon alanine (Araújo et al., 2013), butyrate (Gonçalves et al., 2013) and folic acid (Couto et al., 2012) transport.

Interestingly enough, our results showed that epigallocatechin-3-gallate, quercetin and resveratrol (50 μM), which *per se* did not modify total, GLUT- and non-GLUT-dependent [^{3}H]DG accumulation, completely reversed the inhibitory effect of *tert*-BOOH upon total [^{3}H]DG accumulation. This effect was associated with a complete blockade of the inhibitory effect of *tert*-BOOH upon GLUT-dependent component of [^{3}H]DG accumulation (Fig. 6B).

3.6. Tert-BOOH does not alter glucose metabolism

The amount of extracellular lactate, a product of the glycolysis pathway, was similar in control and *tert*-BOOH-treated cells (27.7 ± 0.7 and 29.7 ± 1.3 nmol lactate mg prot^{-1}, respectively, n = 17).

3.7. Tert-BOOH increases the apical-to-basal transepithelial permeability to [^{3}H]DG across BeWo cells

In the last part of this work, we investigated the effect of *tert*-BOOH upon [^{3}H]DG apical-to-basal transepithelial apparent permeability (P_{app}). In agreement with the results shown above, *tert*-BOOH induced a decrease in [^{3}H]DG intracellular content in BeWo cells (Table 1). However, this compound increased the apical-to-basal P_{app} to [^{3}H]DG (Table 1). Moreover, *tert*-BOOH also increased the P_{app} to two markers of paracellular transport: [^{14}C]sorbitol (Table 1) and phenol red (from 100 ± 22% to 308 ± 95% of control, n = 12).

4. Discussion

Cellular oxidative stress arises when the production of reactive oxygen species overwhelms the ability of enzymatic and non-enzymatic antioxidant defense systems to maintain reactive oxygen species within physiological levels (Kohen and Nyska, 2002).

In this work, we hypothesized that oxidative stress would alter the placental transport of glucose, which is an important determinant of fetal growth and future health of the newborn (Jansson et al., 2009; Vo and Hardy, 2012).

Our results showed that a 24 h-exposure of BeWo cells, a human STB cell model, to 100 μM *tert*-BOOH increased GSX and GSSG levels. Glutathione is a major intracellular antioxidant (Rossi

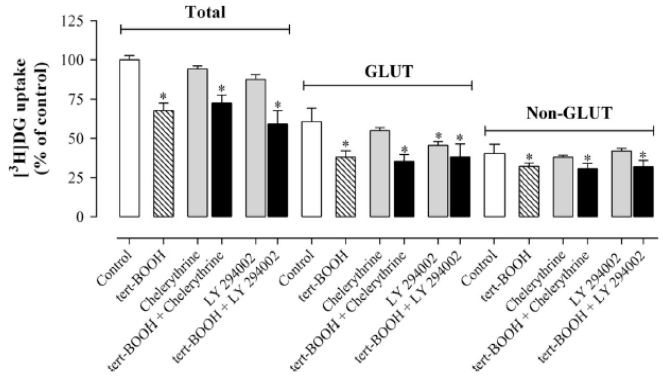

Fig. 5. Effect of inhibitors of intracellular signaling pathways upon the inhibitory effect of *tert*-BOOH on total, GLUT and non-GLUT ^{3}H-2-deoxyglucose ([^{3}H]DG) accumulation by BeWo cells. Cells previously exposed to *tert*-BOOH (100 μM; 24 h) or to its solvent, were incubated at 37 °C with 50 nM [^{3}H]DG for 120 min, at pH 7.4, in the absence or presence of LY-294002 1 μM or chelerythrine 0.1 μM (n = 10–12). Shown are arithmetic means + S.E.M. *Significantly different from the respective control (P < 0.05).

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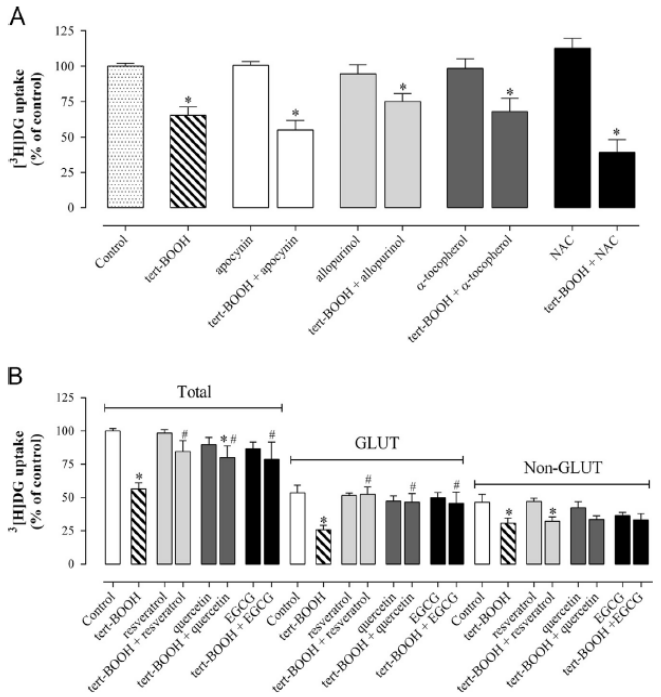

Fig. 6. Effect of antioxidants upon the inhibitory effect of *tert*-BOOH on total ^{3}H-deoxyglucose ([^{3}H]DG) accumulation by BeWo cells. Cells previously exposed to *tert*-BOOH (100 μM; 24 h) or to its solvent, were incubated at 37 °C with 50 nM [^{3}H]DG for 120 min, at pH 7.4, in the absence or presence of (a) inhibitors of reactive oxygen species-generating enzymes: apocynin (1 mM), allopurinol (1 mM), α-tocopherol (1 mM), and *N*-acetyl-cysteine (NAC; 0.1 mM), and (b) polyphenols: resveratrol (50 μM), quercetin (50 μM) or epigallocatechin-3-gallate (EGCG; 50 μM). (n=8–12). Shown are arithmetic means+S.E.M. *Significantly different from the respective control ($P<0.05$) and #significantly different from *tert*-BOOH ($P<0.05$).

Table 1
Apical-to-basal apparent permeability (P_{app}) and intracellular accumulation of [^{3}H]deoxyglucose and [^{14}C]sorbitol in BeWo cells treated with *tert*-BOOH (100 μM; 24 h).

	P_{app} (pmol s^{-1})		P	Intracelullar content (pmol mg prot^{-1})		P
	Control	*tert*-BOOH		Control	*tert*-BOOH	
[^{3}H]deoxyglucose	0.0015 ± 0.00009	0.0026 ± 0.00014	0.000	1.67 ± 0.35	0.60 ± 0.24	0.026
[^{14}C]sorbitol	0.00056 ± 0.00008	0.00093 ± 0.0001	0.015	0.1 ± 0.06	0.07 ± 0.02	0.014

Values represent mean ± S.E.M (n=8–9).

et al., 2006), and an increased intracellular accumulation of GSSG is considered a reliable biomarker of oxidative stress (Rossi et al., 2006). Exposure of BeWo cells to *tert*-BOOH also increased lipid peroxidation and protein carbonylation levels, which constitute two widely accepted markers of oxidative damage to lipids and proteins (Dalle-Donne et al., 2006). As a whole, these results indicate that BeWo cells exposed to 100 μM *tert*-BOOH for 24 h constitute a good cellular model of placental oxidative stress. Elevated lipid peroxidation and protein carbonylation and altered levels of glutathione have also been found in placentae from women with pregnancy disorders associated with oxidative stress (please see Section 1), which is considered an important contributing factor for fetal programming of adult diseases (Giussani et al., 2012). Since the molecular mechanisms underlying these effects of oxidative stress are still largely unexplored (Thompson and Al-Hasan, 2012), our aim was to investigate the effect of *tert*-BOOH (100 μM; 24 h) upon [^{3}H]DG (a glucose analog) uptake and transepithelial permeability in BeWo cells.

Our results showed that *tert*-BOOH reduced the intracellular accumulation of [^{3}H]DG in BeWo cells via both GLUT- and non-GLUT-mediated transports. As described in previous works (Araújo et al., 2008; Shah et al., 1999), [^{3}H]DG uptake by BeWo cells mainly involves a Na^{+}-independent and insulin-insensitive transporter. This most probably corresponds to GLUT1, which is functionally the most relevant glucose transporter present in the STB transporting epithelium (Baumann et al., 2002), although the contribution of other GLUTs, more specifically GLUT3, cannot be excluded. However, it is still controversial whether GLUT3 is expressed

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(Brown et al., 2011) or not (Shah et al., 1999) in BeWo cells. Concerning non-GLUT-mediated [^{3}H]DG uptake, this could correspond to adsorption of [^{3}H]DG to the cell membrane of BeWo cells.

Our results also demonstrated that the effect of *tert*-BOOH was not associated with a change in the mRNA level of GLUT1. This suggests that it rather involves changes in protein levels of GLUT1, post-translational GLUT1 modifications, such as oxidation (Burton and Jauniaux, 2011; Fiorentini et al., 1999) phosphorylation (Han et al., 2006), nitration (Webster et al., 2008) and ubiquitination (Fernandes et al., 2011), or changes in GLUT1 intrinsic activity. These parameters should be accessed in a future work in order to increase knowledge about the impact of *tert*-BOOH-induced oxidative stress upon GLUT1-mediated transport.

We also verified that the inhibitory effect of *tert*-BOOH upon [^{3}H]DG accumulation was not associated with an alteration in glycolytic metabolism, as assessed by measurement of lactate production. Since the first enzyme of glycolysis (hexokinase) is of high affinity (Moraes and Reithmeier, 2012), the reduction in [^{3}H]DG accumulation caused by *tert*-BOOH was probably not sufficiently marked to elicit a decrease in the glycolysis rate.

Very few studies relating oxidative stress with placental glucose uptake have been performed. Interestingly and according to our results, Lappas et al. (2012) and Li et al. (2004) showed that oxidative stress-generating agents reduced placental glucose uptake, although this effect was associated with a reduction in GLUT1 gene expression. These different findings can be explained by the use of distinct oxidative stress-generating agents in these works.

Alterations in placental transport or intracellular metabolism of glucose have been described in some pregnancy disorders associated with increased reactive oxygen species generation, namely fetal growth restriction (Magnusson et al., 2004), gestational diabetes and chronic hypoxia (which is present in preeclampsia) (Baumann et al., 2002; Illsley, 2000). However, placental glucose transport and lactate production are differently altered in these pathologies, suggesting that besides oxidative stress, other factors play an important role in determining the rate of placental glucose transport and metabolism observed in these pathologies.

PI3K and PKC signaling pathways are important regulators of glucose transport (Fernandes et al., 2011; Han et al., 2006; Lappas et al., 2012; Riley et al., 2005; Wieman et al., 2007). Moreover, activation of both of these pathways as a consequence of *tert*-BOOH-induced cellular oxidative stress has also been demonstrated (Li et al., 2011; Perez et al., 2006). Therefore, we decided to clarify the role of these two signaling pathways in the inhibitory effect of *tert*-BOOH upon [^{3}H]DG accumulation by BeWo cells. The results indicated that neither PI3K nor PKC activation seemed to be involved in this effect. Interestingly, the results of PI3K agree with those of a very recent paper, also relating oxidative stress with placental DG uptake (Lappas et al., 2012).

Antioxidants are compounds that inhibit or delay the production of reactive oxygen species and the consequent oxidation of biomolecules (Al-Gubory et al., 2010). These compounds have been used as supplements to counteract oxidative stress and improve fetal health outcomes in females with, or at higher risk of developing, preeclampsia (Chappell et al., 1999), fetal growth restriction (Rueda-Clausen et al., 2012) and gestational diabetes (Cederberg et al., 2001). So, we decided to investigate the ability of different antioxidants to revert *tert*-BOOH-induced inhibition of [^{3}H]DG accumulation. Interestingly, we verified that the polyphenols quercetin, epigallocatechin-3-gallate and resveratrol totally abolished the reduction in [^{3}H]DG accumulation induced by *tert*-BOOH, by specifically blocking the effect of *tert*-BOOH upon GLUT-dependent [^{3}H]DG accumulation. Since these compounds alone did not interfere with GLUT-dependent [^{3}H]DG accumulation, we believe that the effect of polyphenols does not involve a direct interaction with glucose transporters.

Dietary polyphenols possess antioxidant properties due to their ability to scavenge reactive oxygen species, modulate transcription factors, and induce histone modifications (Mitjavila and Moreno, 2012). A previous work from our group showed that the inhibitory effect of quercetin upon *tert*-BOOH-induced reduction of butyrate uptake in intestinal cells is associated with its capacity to abolish lipid peroxidation (Gonçalves et al., 2013). Moreover, resveratrol is able to reverse the decrease in placental GLUT1 activity evoked by hypoxanthine/xanthine oxidase-induced oxidative stress via sirtuin 1, an enzyme with histone deacetylase activity (Lappas et al., 2012). Finally, GLUTs can be upregulated trough a hypoxia-inducible transcriptional factor-1(HIF-1)-mediated pathway in BeWo cells (Baumann et al., 2007), and epigallocatechin-3-gallate (Mandel et al., 2008), quercetin (Bach et al., 2010) and resveratrol (Lin et al., 2012) increase HIF-1 activity and/or expression. Altogether, these observations suggest that polyphenols may exert a protective role against oxidative stress-induced inhibition of placental glucose transport through distinct antioxidant mechanisms.

The reactive oxygen species scavengers α-tocopherol and *N*-acetyl-L-cysteine, the NADPH oxidase inhibitor apocynin and the xanthine oxidase inhibitor allopurinol were not able to reverse the inhibitory effect of *tert*-BOOH upon [^{3}H]DG accumulation. These results suggest that the inhibitory effect of *tert*-BOOH upon DG accumulation does not seem to greatly depend upon reactive oxygen species generation. Curiously, other works also reported that *N*-acetyl-L-cysteine and α-tocopherol were not able to alter oxidative stress-induced reduction of DG transport (Fernandes et al., 2011; Lappas et al., 2012). This can be due to the fact that, in the case of endogenous antioxidants such as vitamin E and glutathione (for which *N*-acetyl-L-cysteine is a precursor (Gallo et al., 2010)), the interaction of multiple systems is necessary to counteract oxidative processes. For instance, the antioxidant activity of vitamin E against lipid peroxidation needs to be supported by the activity of GSH and vitamin C (Gallo et al., 2010).

Fetal glucose availability does not solely rely on placental transport across the apical membrane of the STB. Indeed, changes in glucose transport across the basal membrane of the STB will also have a significant impact upon transplacental glucose transport (Baumann et al., 2002). So, in the last part of this work, we investigated the effect of *tert*-BOOH upon [^{3}H]DG transepithelial permeability in the apical-to-basal direction in BeWo cells. The results obtained showed that, although *tert*-BOOH decreased the intracellular accumulation of [^{3}H]DG in BeWo cells, it significantly increased the transepithelial permeability of [^{3}H]DG in the apical-to-basal direction. Moreover, *tert*-BOOH increased the apical-to-basal transport of the paracellular markers [^{14}C]sorbitol and phenol red. Noteworthy, in intestinal epithelial cells, *tert*-BOOH was recently found to decrease the expression (Kim et al., 2012) and to alter the phosphorylation pattern (Sheth et al., 2009) of the tight junction proteins zonula occludens-1 and occludin, in association with an increase in paracellular transport activity. So, we suggest that *tert*-BOOH, by affecting BeWo cell tight junctions, induces an increase in [^{3}H]DG paracellular transport, and that this effect contributes to the observed increase in transepithelial permeability to [^{3}H]DG.

In conclusion, our work demonstrates that *tert*-BOOH-induced oxidative stress in BeWo cells decreased DG accumulation, and that this effect was completely reversed by some dietary polyphenols. Moreover, *tert*-BOOH-induced oxidative stress increased the transepithelial permeability to DG across BeWo cell monolayers, by increasing its paracellular transport. As a whole, we suggest that oxidative stress reduces placental accumulation of glucose associated with an increase in its transepithelial permeability. This effect may contribute to the deleterious consequences of pregnancy disorders associated with oxidative stress.

Please cite this article as: Araújo, J.R., et al., Oxidative stress induced by *tert*-butylhydroperoxide interferes with the placental transport of glucose: *in vitro* studies with BeWo cells. Eur J Pharmacol (2013), http://dx.doi.org/10.1016/j.ejphar.2013.10.023

Acknowledgments

This work was financially supported by Fundação para a Ciência e a Tecnologia (FCT) and COMPETE, QREN and FEDER (PTDC/SAU-OSM/102239/2008, SFRH/BD/63086/2009 and SFRH/BPD/40170/2007).

The authors wish to thank Prof. João T. Guimarães (Department of Clinical Pathology, Centro Hospitalar S. João, Porto, Portugal) for lactate quantification.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2013.10.023.

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Please cite this article as: Araújo, J.R., et al., Oxidative stress induced by *tert*-butylhydroperoxide interferes with the placental transport of glucose: *in vitro* studies with BeWo cells. Eur J Pharmacol (2013), http://dx.doi.org/10.1016/j.ejphar.2013.10.023

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Reproductive Toxicology 40 (2013) 76–81

Contents lists available at SciVerse ScienceDirect

Reproductive Toxicology

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Oxidative stress decreases uptake of neutral amino acids in a human placental cell line (BeWo cells)

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ARTICLE INFO

Article history:
Received 24 April 2013
Received in revised form 29 May 2013
Accepted 15 June 2013
Available online 24 June 2013

Keywords:
Oxidative stress
Placenta
Transport
Nutrients

ABSTRACT

Increased oxidative stress (OS) is implicated in the pathophysiology of several pregnancy disorders. We aimed to investigate the effect of *tert*-butylhydroperoxide (TBHP)-induced OS upon the placental transport of the neutral amino acids L-methionine (L-Met) and L-alanine (L-Ala), by using a human trophoblast cell model (BeWo cells). TBHP reduced both total and Na^+-independent ^{14}C-L-Met intracellular steady-state accumulation over time (A_{max}), by reducing non-system L-mediated uptake – most probably system y^+ – while having no effect on system L. Moreover, TBHP reduced total ^{14}C-L-Ala A_{max} through an inhibition of system A. The effect of TBHP upon total, but not system A-mediated, ^{14}C-L-Ala uptake was dependent upon phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC) activation, and was completely prevented by the polyphenol quercetin. In conclusion, a reduction in placental uptake of neutral amino acids may contribute to the deleterious effects of pregnancy disorders associated with OS.

1. Introduction

The placenta constitutes the main interface between maternal and fetal blood circulations, and one of its major functions is to mediate the transfer of nutrients from the mother to the fetus, which occurs at the syncytiotrophoblast (STB) epithelium [1]. Placental nutrient transport is absolutely necessary for fetal development, and changes in the activity of specific nutrient (e.g. amino acids) transporters in the human STB have been demonstrated in both growth restriction [2,3] and overgrowth [4]. However, it is still unknown whether these changes are a cause or rather a consequence of the aberrant fetal growth [5].

Amino acids constitute the building blocks for fetal protein synthesis, and are potent stimulators of fetal secretion of insulin, which is the primary growth-promoting hormone during fetal life [6]. At the STB, transport of neutral amino acids occurs primarily via systems A and L [7]. System A is a Na^+-dependent transporter that mediates the uptake of mainly non-essential and short-chain amino acids such as L-alanine (L-Ala), and system L is a Na^+-independent transporter mainly involved in cellular uptake of essential, branched-chain and aromatic amino acids such as L-methionine (L-Met) [6]. L-Ala [8] and L-Met [9] have been used as model substrates to study the activity of systems A and L in the human placenta, respectively.

Cellular oxidative stress (OS) arises when the production of reactive oxygen species (ROS) overwhelms the enzymatic and non-enzymatic antioxidant defense systems that maintain ROS within physiological levels [10]. ROS play an important physiological role as second messengers in intracellular signaling pathways aimed at maintaining cell homeostasis [11,12]. However, at heightened levels, OS can cause damage to several biological molecules [11], leading to loss of function and ultimately to cell death [13].

Considerable evidence implicates increased OS in the pathophysiology and progression of prevalent pregnancy complications such as miscarriage and preeclampsia [14], fetal growth restriction [15] and both gestational [16] and type 1 [17] diabetes mellitus. Interestingly enough, alterations in placental amino acids transport have been associated with most of these disorders [2,4,18]. Moreover, an increased OS intrauterine environment has been demonstrated to program the fetus to develop metabolic and cardiovascular diseases later in life [19]. Since the molecular mechanisms underlying the pathological effects of OS during pregnancy are only beginning to be addressed [20], we aimed to investigate the effect of OS upon the placental uptake of L-Met and L-Ala, by using a human choriocarcinoma cell line (BeWo cells). This cell line, which

Abbreviations: A_{max}, accumulation at steady state; BCH, 2-amino-2-norbornanecarboxylic acid; EGCG, epigallocatechin-3-gallate; k_{in}, rate constant for inward transport; k_{out}, rate constant for outward transport; LAT, L-type amino acid transporter; MeAIB, α-(methylamino)isobutyric acid; NEM, *N*-ethylmaleimide; OS, oxidative stress; SNAT, Na^+-coupled neutral amino acid transporter; STB, syncytiotrophoblast; TBHP, *tert*-butylhydroperoxide.

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0890-6238/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.reprotox.2013.06.073

displays many characteristics of third-trimester trophoblasts [21], is suitable to investigate the placental transport of neutral amino acids, as the expression of transport systems for these nutrients [22,23] is similar to human term placentas [7,24].

2. Materials and methods

2.1. Reagents

L-[1-^{14}C]-Methionine – specific activity 40–60 mCi/mmol – and L-[U-^{14}C]-Alanine – specific activity 56 mCi/mmol – (American Radiolabeled Chemicals, St. Louis, MO, USA); BCH (2-amino-2-norbornanecarboxylic acid), albumin from bovine serum, chelerythrine chloride, decane, EGCG [(−) epigallocatechin-3-gallate], fetal calf serum, Ham's F12K medium (Kaighn's modification), H-89 dihydrochoride hydrate, L-lysine monohydrochloride, LY-294002 (2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), MeAIB (α-(methylamino)isobutyric acid), *N*-acetyl-L-cysteine, NEM (*N*-ethylmaleimide), PD 98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), SP 600125 (1,9-Pyrazoloanthrone, Anthrapyrazolone), quercetin dihydrate, resveratrol and TBHP (*tert*-butyl hydroperoxide solution) (Sigma, St. Louis, MO, USA); DMSO (dimethylsulfoxide) (Merck, Darmstadt, Germany); SB 203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; Alomone Labs Ltd., Jerusalem, Israel).

The final concentration of the solvents in the buffer and culture medium was 1% (v/v).

2.2. BeWo cell culture

The BeWo cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ GmbH, ACC-458). Cells were used between passage numbers 33 and 54 and with seven to eight days after the initial seeding, so that they could spontaneously differentiate and form a confluent and functional STB-like structure [25].

2.3. Treatment with tert-butylhydroperoxide (TBHP)

BeWo cells were exposed to 100 μM of the oxidizing and free radical generating agent TBHP [13] for 24 h in fetal calf serum-free culture media. We recently verified that this treatment induces an increase in OS biomarkers (total and oxidized glutathione, lipid peroxidation products and protein carbonyl groups) – similarly to what is found in placentas obtained from pregnancies with gestational diabetes [26,27], fetal growth restriction [28] and preeclampsia [29,30] – while maintaining cellular viability and proliferation [31]. In contrast, exposure of BeWo cells to concentrations of TBHP higher than 100 μM (300–1000 μM) significantly decreased cell viability and proliferation [31]. So, 100 μM TBHP (24 h) was used in the present experiments aimed at determining the effect of OS upon L-Ala and L-Met transport in BeWo cells.

2.4. Uptake studies

After exposure to TBHP, BeWo cells were preincubated at 37 °C in buffer with the following composition: 125 mM NaCl (unless otherwise stated), 4.8 mM KCl, 1.2 mM KH_2PO_4, 12.5 mM HEPES-NaOH, 12.5 mM MES, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$ and 5.6 mM D(+)-glucose, pH 7.5) for 20 min and incubation was then initiated by the addition of buffer containing 250 nM ^{14}C-L-Met or ^{14}C-L-Ala. Amino acid uptake was stopped by rinsing the cells with ice-cold buffer, and then cells were solubilized with 0.1% (v/v) Triton X-100 (in 5 mM Tris–HCl, pH 7.4). Radioactivity in the cells was measured by liquid scintillation counting and normalized for total cell protein, which was determined as described [32].

Table 1
Primer sequences and annealing temperatures (AT) used for real time RT-PCR.

Gene name	Primer sequence (5′–3′)	AT (°C)
HPRT[a]	Fwd[d]: GGT CAA GGT CGC AAG C Rev[e]: GGG CAT ATC CTA CAA CAA ACT	65
SNAT1[b]	Fwd: ACT ACC CTC TGC CAT AAA Rev: TAT AGC CAA GAT ACC CTA AGT	60
SNAT2[c]	Fwd: GTC ATT GGT GGT CAT TCT T Rev: GTG GTG TTT ATT GTT TCG TTA	60

[a] Hypoxanthine-guanine phosphoribosyltransferase.
[b] Sodium-coupled neutral amino acid transporter 1.
[c] Sodium-coupled neutral amino acid transporter 2.
[d] Forward.
[e] Reverse.

TBHP and transport inhibitors were present during both the preincubation and incubation periods. Inhibitors of intracellular signaling pathways and antioxidants were present throughout the experiment together with TBHP (i.e. 24 h plus preincubation (20 min) and incubation (30 min) periods). Controls were run in the presence of the respective solvents.

TBHP and inhibitors of intracellular signaling pathways (LY-294002 1 μM, H-89 1 μM, chelerythrine 0.1 μM, PD 98059 2.5 μM, SB 203580 10 μM and SP 600125 5 μM) or antioxidants (*N*-acetyl-L-cysteine (NAC), quercetin, epigallocatechin-3-gallate (EGCG) and resveratrol, all at 50 μM) alone or in combination with TBHP did not alter cell viability (results not shown).

System A-mediated ^{14}C-L-Ala uptake was measured as the MeAIB (2 mM)-sensitive component of total ^{14}C-L-Ala uptake, as previously described [8,33]. Non-system A-mediated ^{14}C-L-Ala uptake was calculated as the MeAIB (2 mM)-insensitive component of total ^{14}C-L-Ala uptake.

System L-mediated ^{14}C-L-Met uptake was measured as the Na^+-independent and BCH (2 mM)-sensitive component of ^{14}C-L-Met uptake, and non-system L-mediated ^{14}C-L-Met uptake was calculated as the Na^+-independent and BCH (2 mM)-insensitive component of ^{14}C-L-Met uptake. Na^+-independent uptake was measured by incubating cells in buffer in which 125 mM NaCl was isotonically replaced by LiCl.

2.5. RNA extraction and real-time RT-PCR (qRT-PCR)

RNA extraction, cDNA synthesis and qRT-PCR were performed in BeWo cells as described by Araujo et al. [34]. Annealing temperature and sequence of primers used are listed in Table 1. The amount of mRNA of each tested gene was normalized to the amount of mRNA of the housekeeping gene (hypoxanthine-guanine phosphoribosyltransferase). TBHP did not affect the expression levels of hypoxanthine-guanine phosphoribosyltransferase (results not shown).

2.6. Calculations and statistics

The analysis of time-course and saturation curves of ^{14}C-L-Ala or ^{14}C-L-Met uptake was performed by using a non-linear regression analysis, as previously described by our group [35].

Arithmetic means are given with standard error of the mean (S.E.M). Statistical significance of the difference between various groups was evaluated by one-way analysis of variance followed by the Student–Newman–Keuls *post* test. For comparison between two groups, Student's *t*-test was used. Differences were considered to be significant when $P < 0.05$.

The value of *n* indicates the number of experiments.

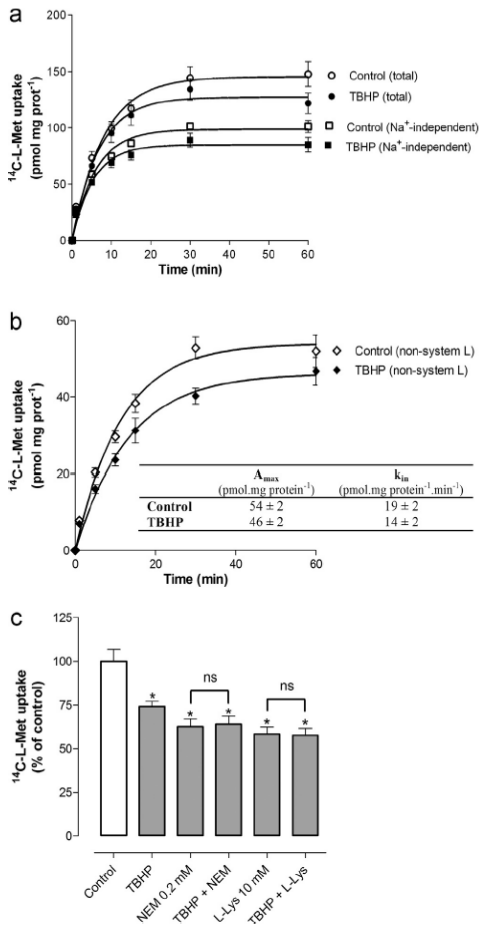

Fig. 1. Effect of TBHP (24 h; 100 μM) upon ^{14}C-L-Met uptake by BeWo cells. (a, b) Time-course of uptake of 250 nM ^{14}C-L-Met at 37 °C, pH 7.5, in buffer containing Na^+ (*total; a*) or in Na^+-free buffer in the absence (*Na^+-independent; a*) or presence of BCH (*non-system L; b*) (n = 6–8). (c) Effect of inhibitors or substrates of system y^+ upon non-system L-mediated ^{14}C-L-Met accumulation, determined in cells incubated at 37 °C with 250 nM ^{14}C-L-Met for 30 min, at pH 7.5, in Na^+-free buffer containing BCH 2 mM, in the absence or presence of *N*-ethylmaleimide (NEM) 0.2 mM or L-Lysine (L-Lys) 10 mM (n = 6–10). Analysis of the time course allowed the determination of the rate constant of inward transport (k_{in}) and the steady state accumulation (A_{max}). Shown are arithmetic means ± S.E.M. *Significantly different from the respective control (P < 0.05); ns, not significantly different.

3. Results

3.1. Effect of TBHP upon L-Met uptake by BeWo cells

We first determined the effect of TBHP upon the time-course of ^{14}C-L-Met uptake, by incubating BeWo cells with ^{14}C-L-Met (250 nM) for various periods of time (Fig. 1). Our results showed that TBHP reduced total and Na^+-independent ^{14}C-L-Met steady-state accumulation (A_{max}) (from 145 ± 6 to 127 ± 5 and from 99 ± 2 to 85 ± 3 pmol mg prot^{-1}, respectively) (Fig. 1a).

Na^+-independent ^{14}C-L-Met uptake involved a BCH-sensitive transporter (system L) and a BCH-insensitive transporter (designated as non-system L), having a similar contribution to total ^{14}C-L-Met uptake (A_{max} of 48 ± 1 pmol mg prot^{-1} for system L and 54 ± 2 pmol mg prot^{-1} for non-system L). The effect of TBHP was associated with a decrease in non-system L-component of ^{14}C-L-Met uptake, as TBHP decreased both the A_{max} and the rate constant of inward transport (k_{in}) of ^{14}C-L-Met uptake mediated by this component (Fig. 1b). System L-mediated ^{14}C-L-Met uptake was not affected by TBHP (results not shown).

So, in the next series of experiments, we further characterized the effect of TBHP upon non-system L-mediated ^{14}C-L-Met uptake. First, we investigated its effect upon the kinetic parameters of non-system L-mediated ^{14}C-L-Met uptake. Our results revealed that neither K_m (142 ± 47 μM) nor V_{max} (6.7 ± 1.7 nmol mg prot^{-1} 5 min^{-1}) (n = 6) values were significantly affected by TBHP (results not shown). Then, because we recently reported that besides system L, a Na^+-independent and BCH-insensitive transporter, which we hypothesized to be system y^+, appears to participate in ^{14}C-L-Met uptake by BeWo cells [34], we decided to investigate the effect of the system y^+ inhibitor NEM [36,37] and of the system y^+ substrate L-Lys [38] upon ^{14}C-L-Met accumulation. As shown in Fig. 1c, ^{14}C-L-Met accumulation was inhibited by NEM and by L-Lys under control conditions, suggesting the involvement of system y^+ in ^{14}C-L-Met uptake. Additionally, our results also suggest that TBHP probably reduced system y^+-mediated ^{14}C-L-Met accumulation, because the inhibitory effect of TBHP disappeared in the presence of NEM or L-Lys, which inhibit system y^+ (Fig. 1c).

3.2. Effect of TBHP upon L-Ala uptake by BeWo cells

3.2.1. Effect upon ^{14}C-L-Ala uptake

The effect of TBHP upon the time-course of ^{14}C-L-Ala uptake is shown in Fig. 2a. TBHP was found to reduce the A_{max} of both total and system A-mediated ^{14}C-L-Ala uptake (total uptake: from 83.1 ± 5.7 to 63.5 ± 3.0 pmol mg prot^{-1}; and system A-mediated uptake: from 23.0 ± 3.3 to 7.2 ± 1.1 pmol mg prot^{-1}). On the other hand, non-system A-mediated ^{14}C-L-Ala uptake was not affected (results not shown).

Our next step was to investigate the intracellular signaling pathways involved in the effect of TBHP upon total and system A-mediated ^{14}C-L-Ala uptake, by assessing the effect of exposing BeWo cells to inhibitors of some intracellular signaling pathways, to TBHP or both. Based on previous works from our group [34,39], the signaling pathways studied were phosphoinositide 3-kinase (PI3K)), protein kinases A (PKA) and C (PKC), and members of the mitogen-activated protein kinases (MAPK): extracellular-signal-regulated-kinase 1/2 (ERK/MEK 1/2), Jun-NH2-terminal kinase (JNK) and p38 MAPK. All of these pathways have been shown to be modulated by OS [12,40] and to affect system A activity [41].

Total ^{14}C-L-Ala uptake was reduced (by 10–15%) in the presence of a JNK inhibitor (SP 600125 5 μM), indicating that JNK activation may be required for the basal uptake of this amino acid (Fig. 2b). On the other hand, the inhibitory effect of TBHP upon total ^{14}C-L-Ala uptake was reversed by LY-294002 1 μM and chelerythrine 0.1 μM (specific inhibitors of PI3K and PKC, respectively), but not by SP 600125, suggesting that the effect of TBHP depends upon PI3K and PKC, but not JNK, activation (Fig. 2b). In contrast, inhibition of PKA (with H-89 1 μM), ERK1/2 (with PD 98059 2.5 μM) or p38 MAPK (with SB 203580 10 μM) did not alter neither total ^{14}C-L-Ala uptake nor the effect of TBHP upon it (Fig. 2b).

We also verified that basal system A-mediated ^{14}C-L-Ala uptake appears to depend on p38 MAPK and JNK activation, because specific inhibitors of these pathways reduced system A-mediated ^{14}C-L-Ala uptake (by about 15%). However, none of the

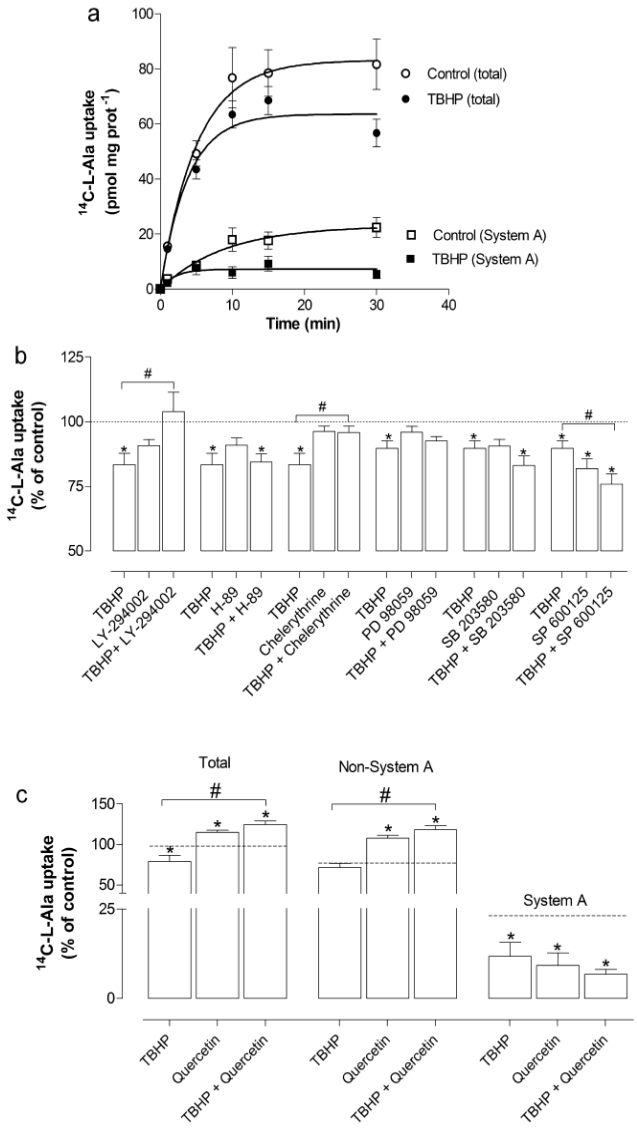

Fig. 2. Effect of TBHP (24 h; 100 μM) upon ^{14}C-L-Ala uptake by Bewo cells. (a) Time-course of uptake of 250 nM ^{14}C-L-Ala at 37 °C, pH 7.5, in the absence (*total; a*) or presence of MeAIB 2 mM (*system A; a*) (n = 6–8). (b) Effect of inhibitors of intracellular signaling pathways upon total ^{14}C-L-Ala accumulation, determined in cells incubated at 37 °C with 250 nM ^{14}C-L-Ala for 30 min, at pH 7.5, in the absence or presence of LY-294002 1 μM, H-89 1 μM, chelerythrine 0.1 μM, PD 98059 2.5 μM, SB 203580 10 μM or SP 600125 5 μM (n = 11–15). (c) Effect of quercetin upon total, non-system A and system A-mediated ^{14}C-L-Ala accumulation, determined in cells incubated at 37 °C with 250 nM ^{14}C-L-Ala for 30 min, at 37 °C, pH 7.5, in the absence or presence of quercetin 50 μM (n = 12). Shown are arithmetic means ± S.E.M. *Significantly different from the respective control (total, non-system A or system A mediated-uptake) represented in dashed lines (P < 0.05). #Significantly different from TBHP (P < 0.05).

above-mentioned inhibitors reversed the effect of TBHP upon system A-mediated ^{14}C-L-Ala uptake (results not shown).

We also investigated the ability of some antioxidants – the free radical scavenger and glutathione precursor NAC [42], and the polyphenols EGCG, quercetin and resveratrol [43] – to prevent the inhibitory effect of TBHP upon total and system A-mediated ^{14}C-L-Ala uptake. Apart from quercetin, none of the compounds tested were able to interfere with the inhibitory effect of TBHP upon total ^{14}C-Ala uptake (results not shown). As for quercetin, this compound alone increased total ^{14}C-L-Ala uptake (by 15%; Fig. 2c), which was associated with a strong (±30%; Fig. 2c) stimulatory effect upon non-system A-mediated component of uptake.

Unexpectedly, quercetin was able to completely prevent the inhibitory effect of TBHP upon total ^{14}C-Ala uptake, by increasing non-system A-mediated uptake, while having no effect upon system A-mediated ^{14}C-Ala uptake (Fig. 2c).

3.2.2. Effect upon the mRNA levels of system A transporters

Na^+-coupled neutral amino acid transporters (SNAT) 1 and 2 are the major proteins responsible for system A activity and expression in STB at term [8,44] and in BeWo cells [24,45]. By qRT-PCR, we verified that the mRNA expression levels of SNAT1 and SNAT2 were similar in both control and TBHP-treated cells (results not shown).

4. Discussion

In this work, we hypothesized that OS may impair the placental transport of neutral amino acids, which are important determinants of fetal growth [6] and future health of the newborn [1]. So, we aimed to investigate the effect of an OS inducer (TBHP) upon ^{14}C-L-Met and ^{14}C-L-Ala uptake by BeWo cells.

OS was induced with TBHP, which is known to increase lipid peroxidation and protein carbonylation levels and decrease antioxidant (glutathione) capacity in BeWo cells [31]. Importantly, similar oxidative modifications have been found in placentas and maternal circulation in pregnancy disorders associated with increased OS, namely miscarriage [14], preeclampsia [29,30,46], fetal growth restriction [15,28,47] and gestational diabetes [16,17,26,27].

Our results showed that TBHP reduced total and Na^+-independent ^{14}C-L-Met uptake by decreasing non-system L-mediated uptake, while it did not affect system L-mediated uptake. Confirming our previous hypothesis [34], non-system L-mediated ^{14}C-L-Met uptake by BeWo cells most probably corresponds to system y^+. This is supported by the Na^+-independence, by the NEM and L-Lys-sensitivity and by its similar affinity ($K_m \pm 142\,\mu M$) to two system y^+ isoforms ($K_m \pm 100$–$400\,\mu M$) [48]. Interestingly, TBHP appears to reduce system y^+-mediated ^{14}C-L-Met uptake.

One probable explanation for the contribution of OS to fetal programming involves alterations in DNA methylation [20], which depend on the bioavailability of methyl carriers such as L-Met [1]. TBHP is known to generate methyl radicals [49] which may induce hypermethylation of DNA bases [50]. This epigenetic modification may act as a trigger to reduce the placental uptake of L-Met, thereby decreasing its intracellular levels. So, the observed decrease in placental transport of ^{14}C-L-Met, probably via a reduction in system y^+ activity, may provide a potential link between OS, placental function and fetal programming.

Our results also showed that TBHP reduced total ^{14}C-L-Ala uptake by decreasing system A-mediated uptake, while not affecting either SNAT1 or SNAT2 mRNA expression. These results suggest that increased OS might have consequences for fetal growth by decreasing placental system A activity. In agreement with our findings, fetal growth restriction – which has been associated with oxidative damage in trophoblasts [15] – is correlated with reduced placental system A activity [2], although no changes in SNAT1 or SNAT2 mRNA expression levels were found [51].

Interestingly enough, previous works using other OS-inducing agents and different models of human STB also described that OS inhibits system A [8,52,53] but not system L-mediated transport [8,52]. So, TBHP-treated BeWo cells appear to be a good paradigm to study the effect of OS upon placental amino acid transport. The fact that BeWo cells have clear advantages over other STB models, such as greater stability, life-span, viability with passage, easier maintenance [54] and absence of patient variability, reinforces even more the suitability of this cell model to study the effect of OS upon placental transport function.

One important consequence of cellular OS is the activation of intracellular serine/threonine kinases [12,43]. So, in order to clarify the molecular mechanisms by which TBHP reduced total and system A-mediated ^{14}C-L-Ala uptake in BeWo cells, we searched for the involvement of intracellular signaling pathways. ^{14}C-L-Ala uptake was found to be dependent on JNK (total and system A-mediated) and p38 MAPK (system A-mediated) activation. The results of system A agree with a previous study also performed in BeWo cells [41] except that, in contrast to our observation, Fang et al. found system A activity also to be regulated by PI3K. This discrepancy may be related to the different modes of action of the PI3K inhibitors used (LY-294002 vs. wortmannin).

In this study, we also demonstrated that PI3K and PKC stimulation are involved in the inhibitory effect of TBHP upon total ^{14}C-L-Ala uptake. However, none of the studied intracellular signaling pathway inhibitors (PI3K, PKA, PKC and MAPK) reversed the effect of TBHP upon system A-mediated ^{14}C-L-Ala uptake.

Antioxidants have been demonstrated to counteract OS associated with the pathogenesis of reproductive disorders such as preeclampsia [55], fetal hypoxia [19] and gestational diabetes [16]. So, in the last part of this work, we investigated the ability of NAC and some polyphenols to prevent TBHP-induced inhibition of total and system A-mediated ^{14}C-L-Ala uptake. Of the tested antioxidants, quercetin was able to blunt the reduction in total ^{14}C-L-Ala uptake induced by TBHP (through an increase in non-system A-mediated ^{14}C-L-Ala uptake, which most probably corresponds to system ASC) [56], thereby counteracting the inhibitory effect of TBHP upon system A activity.

Quercetin is one of the polyphenols with the highest antioxidant activity [57] due to its ability to directly scavenge ROS and consequently to decrease the oxidation of biological molecules [43]. Recently, our group demonstrated that the preventive effect of quercetin upon TBHP-induced inhibition of ^{14}C-butyrate uptake was associated with its capacity to abolish lipid peroxidation induced by TBHP [58]. So, the protective role of quercetin against OS-induced inhibition of L-Ala transport is probably related to its antioxidant capacity.

In conclusion, our work demonstrates that TBHP-induced OS in BeWo cells: (a) decreases L-Met uptake through a decrease in non-system L-mediated transport (probably corresponding to system y^+) and (b) decreases L-Ala uptake through a decrease in system A-mediated transport. The effect of TBHP upon total ^{14}C-L-Ala uptake was found to be PI3K- and PKC-dependent and to be prevented by the polyphenol quercetin. We can thus speculate that a reduction in placental transfer of neutral amino acids may contribute to the deleterious implications of increased OS for fetal growth.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by Fundação para a Ciência e a Tecnologia (FCT) and COMPETE, QREN and FEDER (PTDC/SAU-OSM/102239/2008, SFRH/BD/63086/2009 and SFRH/BPD/40170/2007).

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DISCUSSION AND CONCLUSIONS

It is currently accepted that GDM precipitates offspring's risk for developing adverse perinatal outcomes and cardiometabolic [Boney et al., 2005; Gluckman et al., 2008] and neurological [Ornoy, 2005; Stenninger et al., 1998] complications later in life. However, the exact molecular mechanisms underlying this pathological and programming effects are still largely unexplored [Lehnen et al., 2013]. One hypothesis is that GDM affects the maternal-to-fetal transport of nutrients [Metzger et al., 2007] and placental development [Simeoni and Barker, 2009], thereby inducing lifelong changes in gene expression through epigenetic mechanisms.

Nutrient transporters present in the STB epithelium have been suggested to play an important role in fetal programming because: 1) transporters activity and expression constitute an important determinant of fetal growth and development [Jansson et al., 2009]; 2) epigenetic modifications, in particular gene methylation, depend on an adequate provision of nutrients such as folates, L-Met [Jansson et al., 2009], and LC-PUFAs [Kulkarni et al., 2011] (Fig. 2) and changes in placental transport of these nutrients will alter their availability to the fetus, providing a direct link between placental function, gene methylation and fetal programming; and 3) placental nutrient transporters may be themselves key targets for epigenetic modification [Jansson et al., 2009; Jansson and Powell, 2007].

So, considering the important role of nutrient transporters in determining fetal growth and future health of the newborn, the first aim of this work was to investigate if GDM (diagnosed according to [Carpenter and Coustan, 1982]) affected the placental transport of FA, L-Met and essential LC-PUFAs (AA and DHA). For that, we characterized the uptake of ^{3}H-FA, ^{14}C-L-Met, ^{14}C-AA and ^{14}C-DHA by NTB cells and compared it with the uptake by DTB cells.

Human primary cultured cytotrophoblasts are a suitable model to study the placental transport function because they spontaneously differentiate into a functional and polarized STB-like structure that retains all the cellular machinery of the *in vivo* STB [Bischof and Irminger-Finger, 2005; Bloxam et al., 1997; Kliman et al., 1986; Lager et al., 2011]. When comparing ^{3}H-FA transport characteristics in NTB and DTB cells, we found that, although intracellular accumulation of ^{3}H-FA was similar in both cells, DTB cells showed higher rates of inward and outward

transport, suggesting that in GDM placentas a higher turnover of intracellular FA was required to maintain FA homeostasis (**publication A**).

Keating et al. reported that FA uptake by NTB cells is pH-dependent, operating optimally at acidic pH (5.0–5.5), and shows different transport characteristics at acidic (5.5) and physiological (7.5) pH [Keating et al., 2009a]. Similarly to the *in vivo* situation [Solanky et al., 2010], FA uptake into NTB cells at pH 7.5 was shown to involve RFC1, FR-α and PCFT [Keating et al., 2009a], reinforcing the suitability of this cell model to study FA placental transport. In addition, at pH 5.5, RFC1 and PCFT seem to mediate FA apical uptake into NTB cells [Keating et al., 2009a].

In the present work, we showed that ^{3}H-FA uptake by both NTB and DTB cells exhibited similar kinetics and occurred optimally at an acidic pH. However, a greater pH-dependence was observed in DTB cells for low pH values (5.0–6.0), which could indicate a proportionally greater involvement of the high affinity folate:H^+ symporter PCFT. In other words, there seemed to be a higher PCFT:RFC1 relative activity in DTB in comparison with NTB cells (**publication A**).

As a whole, these results suggested that, although quantitatively similar to normal pregnancies, placental transport of FA was more dependent on PCFT than on RFC1 in GDM pregnancies (**publication A**). Since GDM modulates the methylation of genes responsible for fetal growth and energy metabolism at placental level [Lehnen et al., 2013], and methylation of RFC1 gene is associated with a lower expression of this transporter [Farkas et al., 2013], we can speculate that GDM affects FA transport through epigenetic mechanisms.

Comparison of the uptake of L-Met, another nutrient crucial for methylation reactions, between NTB and DTB cells, revealed that ^{14}C-L-Met uptake was quantitatively similar in both cells, in a similar way as described for FA. Specifically, our results showed that ^{14}C-L-Met uptake in both NTB and DTB cells presented a similar profile of time-dependence, kinetics and Na^+-independence (**publication B**). Additionally, system L, more specifically its LAT1 isoform, seemed to greatly contribute (40-60%) to ^{14}C-L-Met uptake in both NTB and DTB cells. System y^+L (possibly corresponding to y^+LAT2 activity) was also functionally present in both NTB and DTB cells, although it had a small contribution (approximately 20%) to ^{14}C-L-Met uptake. On the other hand, some differences

were apparent: systems A and b^{0+} and LAT2 system L isoform were functionally capable of transporting L-Met in DTB cells only, and system y^+L (possibly through the activity of both y^+LAT1 and y^+LAT2) seemed to be more active in DTB compared to NTB cells (**publication B**).

We also investigated if the differences in the uptake characteristics of ^{14}C-L-Met uptake in NTB and DTB cells were associated with differences at the transcriptional level (mRNA) of amino acid transporters. The mRNA levels of SNAT1 and SNAT2 (the major system A isoforms present in STB during late gestation) [Tsitsiou et al., 2011]), and of LAT2 and y^+LAT1 were similar in NTB and DTB cells (**publication B**). System b^{0+} expression was not tested because its function at the MVM and BM of the human STB is still under discussion [Ayuk et al., 2000; Cleal et al., 2011; Cleal and Lewis, 2008; Jansson et al., 2002].

Data available on placental transport of amino acids in GDM pregnancies is conflicting: either an increase [Jansson et al., 2002] or no alteration [Dicke and Henderson, 1988; Nandakumaran et al., 2004] in systems A and L activity and no alterations in the transport of L-Lys (a substrate of systems y^+ and y^+L) [Jansson et al., 2002] have been described. The contrasting findings between these studies (including our own) may be the result of differences in study populations (different maternal body mass index, metabolic control following diagnosis, therapeutics of GDM, GDM diagnostic criteria, incidence of large-for-gestational-age infants and fetal and placental weights).

In conclusion, the results of L-Met uptake showed that GDM does not quantitatively alter L-Met placental transport capacity, although it involved distinct amino acid transporters (LAT1 and y^+LAT2 in NTB cells and LAT1 and 2, y^+LAT1 and 2 and systems A and $b^{0,+}$ in DTB cells) (**publication B**). Given the broad substrate specificities of systems L, y^+L, A and b^{0+} (see Introduction), we suggest that the transport of other neutral amino acids and of cationic amino acids may be altered in GDM pregnancies. Considering this, it will be important to study the placental transport of other amino acids in this disease.

GDM adversely affects behavioral, cognitive and intellectual development of the offspring [Fraser et al., 2012; Larque et al., 2011; Ornoy, 2005] (see Introduction), and low LC-PUFAs concentrations (which are important for fetal neurological and visual development [Innis, 2005]) are observed in placentas

[Pagan et al., 2013] and cord blood [Min et al., 2005; Pagan et al., 2013; Thomas et al., 2005] obtained from GDM pregnancies, indicating that maternal-to-fetal transfer of LC-PUFAs might be impaired. According to this, it seemed of particular importance to compare placental transport of LC-PUFAs in normal and GDM pregnancies.

Uptake of ^{14}C-AA and ^{14}C-DHA by NTB cells was shown to involve both a protein-mediated mechanism - which was quantitatively more important for lower substrate concentrations - and simple diffusion – which was quantitatively more important for higher substrate concentrations (**publication C**). This observation is in agreement with claims of other authors suggesting that under blood physiological concentrations of LC-PUFAs, most of the cellular uptake in peripheral tissues (eg. skeletal muscle, adipose tissue and heart) occurs via a protein-mediated pathway, simple diffusion being quantitatively less important (reviewed by [Bonen et al., 2007; Doege and Stahl, 2006]). In a more detailed characterization of protein-mediated uptake of ^{14}C-AA and ^{14}C-DHA, we could demonstrate that this process greatly depended on the activity of ACSL, as it was markedly inhibited by triacsin C, a potent inhibitor of ACSL activity [Tobin et al., 2009; Tomoda et al., 1991] (**publication C**). On the other hand, the ATP-independence of ^{14}C-AA and ^{14}C-DHA uptake argued against the involvement of FATP in this process (**publication C**). So, we could conclude that ACSL-mediated fatty acid esterification into acyl-CoA was an important mediator of the placental transport of ^{14}C-AA and ^{14}C-DHA (**publication C**). Nevertheless, because ^{14}C-AA and ^{14}C-DHA uptake by NTB cells was strongly (40-50%) though not completely inhibited by triacsin C, we cannot rule out the potential involvement of other transporters in this process, in particular pFABPpm (**publication C**), which has a preferential affinity for AA and DHA and is found exclusively on the MVM of the STB (please see Introduction). However, since pFABPpm protein and its gene sequence has not yet been described, further information on its structure and function is needed before more detailed conclusions can be drawn regarding its involvement in LC-PUFAs placental transport [Gil-Sanchez et al., 2012; Larque et al., 2011].

Uptake of both LC-PUFAs was found to be markedly reduced (≥ 50%) in DTB cells, through a decrease in both protein-mediated uptake and simple diffusion, suggesting a compensatory downregulation of fatty acid transporters at

the placental level in response to high nutritent availability, an hallmark of GDM. LC-PUFAs uptake downregulation was associated with a quantitatively similar reduction of ACSL1 mRNA levels in DTB cells (**publication C**). Both these observations supported the involvement of ACSL1 in the placental transport of ^{14}C-AA and ^{14}C-DHA, and clearly indicated that the decrease in its expression underlined the decrease in ^{14}C-AA and ^{14}C-DHA uptake found in DTB cells. Also important is the fact that the interaction between FATP and ACSL, ie. matched activity and/or expression, which is still a matter of debate [Doege and Stahl, 2006; Richards et al., 2006], did not appear to exist in primary trophoblast cells, according to our results (**publication C**).

By clearly showing a decrease in the placental uptake of LC-PUFAs associated with a decrease in ACSL1 gene expression in GDM, our results may well explain the reduced plasma levels of AA and DHA found in neonates born from women with GDM [Min et al., 2005; Pagan et al., 2013; Thomas et al., 2005; Wijendran et al., 2000], and the neurodevelopmental fetal malprogramming associated with this disease [Pagan et al., 2013].

Due to the crucial role that LC-PUFAs have for fetal visual, behavioral and cognitive development [Cunningham and McDermott, 2009; Duttaroy, 2009], further investigation is needed to elucidate if maternal LC-PUFAs supplementation during pregnancy, which has been proved in a clinical trial to increase fetal levels of LC-PUFAs [Carlson et al., 2013], can overcome GDM-induced adverse health outcomes in the newborn associated with an insufficient fetal supply of LC-PUFAs.

Normal placental development relies on a balanced growth, proliferation, differentiation and programmed cell death of trophoblasts [Huppertz and Herrler, 2005]. Alterations in these properties have been associated with miscarriage [Minas et al., 2007; Pestka et al., 2011], preeclampsia [Arnholdt et al., 1991; Shaker and Sadik, 2013] and FGR [Erel et al., 2001; Ishihara et al., 2002]. Since there is scarce and conflicting knowledge concerning the impact of GDM on these properties [Belkacemi et al., 2013; Sgarbosa et al., 2006], we decided to compare the viability, proliferation, differentiation and apoptosis of both NTB and DTB cells.

A marked and similar increase in proliferation and apoptosis, but no change in viability or differentiation, was observed in DTB, in relation to NTB cells. These

observations suggested that a higher cell turnover may be present in DTB cells as a GDM-related phenotype (**publication C**). Sgarbosa and co-workers also reported an increase in trophoblast apoptosis in GDM, and suggested that hyperglycemia may be a key factor evoking this response [Sgarbosa et al., 2006]. On the other hand, Belkacemi et al. reported a decrease in trophoblast apoptosis with no change in proliferation in GDM placentas [Belkacemi et al., 2005]. These contrasting results, in comparison with ours, can be explained by differences in GDM therapy (women were treated with either diet or diet associated with metformin in Belkacemi's study and in our study women were treated with diet or diet associated with insulin) and placental and neonatal birth weight (which were both higher in GDM compared with control women in Belkacemi's study, but not in ours).

Trophoblast development has been demonstrated to be regulated by GDM-associated hallmarks. In extravillous trophoblasts, we observed that hyperinsulinemia increased proliferation and TNF-α increased viability, although hyperglycemia and hyperleptinemia did not affect these properties (Araújo et al. preliminary results) (**publication C**). In human villous trophoblasts, leptin stimulates proliferation and inhibits apoptosis [Gambino et al., 2012] and TNF-α induces apoptosis [Al-Nasiry et al., 2006; Yui et al., 1994] and proliferation [Yang et al., 1993]. Altogether, these observations suggest that GDM may impair placental development through many of the conditions associated with it.

From the analysis and discussion of the results presented in **chapter I** we can conclude that GDM can disturb the maternal-to-fetal transport of nutrients, particularly of LC-PUFAs, and also trophoblast development. These alterations may eventually contribute to the fetal and postnatal adverse health outcomes associated with GDM.

Oxidative stress is defined as an imbalance in pro-oxidants and antioxidants, in favor of the former [Jones, 2008]. Pregnancy *per se* is considered as a physiological state of oxidative stress [Chen and Scholl, 2005], which is important for embryo implantation, for fetal and placental development, and for labor [Myatt, 2010]. However, at abnormally high levels, oxidative stress can cause damage to macromolecules (inhibiting their normal function [Burton and Jauniaux, 2011])

and disruption of intracellular signaling pathways [Jones, 2008], leading ultimately to cell death [Shaker and Sadik, 2013]. Oxidative stress has been implicated in the pathophysiology of prevalent pregnancy disorders, such as miscarriage [Myatt and Cui, 2004], preeclampsia [Siddiqui et al., 2010], FGR [Son et al., 2004; Takagi et al., 2004] and maternal diabetes (T1D and GDM) [Lappas et al., 2011; Peuchant et al., 2004; Toescu et al., 2004]. This condition has also been demonstrated to program the fetus to develop metabolic and cardiovascular complications later in life [Giussani et al., 2012; Thompson and Al-Hasan, 2012].

In both T1D and GDM, increased levels of oxidative stress have been found in maternal plasma and serum [Peuchant et al., 2004; Rajdl et al., 2005; Toescu et al., 2004], but the placenta have been scarcely studied [Pustovrh et al., 2000]. Due to this, we decided to compare the oxidative stress levels and antioxidant capacity in placentas from GDM, T1D and control women.

From the analysis of our results, we could observe that two widely accepted biomarkers of oxidative damage (malonaldehyde [MDA] and protein carbonyls) [Dalle-Donne et al., 2006] were elevated in T1D, but not in GDM placentas, when compared to controls (**publication D**).

Antioxidants are compounds of enzymatic or non-enzymatic origin that inhibit or delay the production of ROS and the consequent oxidation of biomolecules [Al-Gubory et al., 2010]. Glutathione, the major cellular non-enzymatic antioxidant [Al-Gubory et al., 2010] and the antioxidant enzyme glutathione peroxidase (GPx) [Agarwal et al., 2012] are reliable indicators of antioxidant status [Chen and Scholl, 2005]. When compared to control and GDM placentas, higher reduced (GSH) and lower oxidized (GSSG) glutathione concentrations were found in T1D placentas. On the other hand, GSX, GSH and GSSG levels were similar in GDM and control placentas (**publication D**). Additionally, GPx activity was higher in T1D but not in GDM placentas, in comparison to control ones. These results suggest that a compensatory antioxidant mechanism may develop in T1D placentas to overcome higher oxidative stress levels. In agreement with our results, a parallel increase in blood levels of oxidative stress biomarkers and antioxidant enzymes activity have been reported in T1D pregnant women [Al-Shebly and Mansour, 2012; Orhan et al., 2003]. On the other hand, we found that placental oxidative stress and antioxidants levels were unaltered in GDM, even after stratification for GDM

therapy, mode of delivery and large-for-gestational age newborns (**publication D**). This observation is in contrast to previous studies that associated GDM with increased levels of oxidative stress [Biri et al., 2006; Coughlan et al., 2004; Gelisgen et al., 2011; Toescu et al., 2004]. We believe that the main reason for this apparent inconsistency is that women in our GDM group were diagnosed according to the new IADPSG criteria [Long and Cundy, 2013; Metzger et al., 2010], which encompassed women with a less severe diabetic phenotype. Of note, our GDM women showed third trimester fasting glycemia that is not characteristic of overt diabetes (≤ 5.0 mM) [ADA, 2013; Anger et al., 2012] and glycosylated hemoglobin levels at term that were within the acceptable range for managed diabetes (≤ 5.7 %). In contrast, maternal third trimester fasting glycemia in T1D women was characteristic of overt diabetes (7.9 mM), which was positively correlated with placental MDA levels (**publication D**).

As a whole, the results from **chapter II**: 1) showed that T1D, but not GDM, is associated with increased oxidative stress at the placental level; 2) support the concern that the new IADPSG criteria may be diagnosing as GDM a large number of women that may not in fact be metabolically at high risk [ADA, 2013; Long and Cundy, 2013], with consequences in terms of maternal stress, quality of life and of health care costs [Long and Cundy, 2013]; and 3) support the role of hyperglycemia as an important condition associated with increased oxidative stress in T1D pregnancies [Chen and Scholl, 2005; Lappas et al., 2011; Peuchant et al., 2004].

Altought we did not found increased levels of oxidative stress at placental level in GDM, this condition has been reported to be present in blood of GDM women (see above). Also, increased levels of oxidative stress have been found in placentas obtained from pregnancies complicated by FGR [Karowicz-Bilinska et al., 2004] and preeclampsia [Gulmezoglu et al., 1996; Zusterzeel et al., 2001]. According to this, and considering that glucose is the primary substrate for fetal oxidative metabolism [Hahn et al., 1999], it seemed particularly interesting to investigate if oxidative stress altered the placental transport of glucose (**chapter III, publication E**). To accomplish this, we first characterized the uptake of ^{3}H-2-deoxy-D-glucose (^{3}H-DG) – a D-glucose analogue efficiently transported by GLUT

transporters [Shah et al., 1999] - by the human choriocarcinoma-derived cell line BeWo (**publication E**).

BeWo cells are a well-characterized trophoblast cell model that is widely used to study placental transport of nutrients [Bode et al., 2006; Sastry, 1999]. They show great stability, life-span and viability with passage, easy maintenance and absence of patient variability [Antony et al., 2007; Bode et al., 2006]. Additionally, BeWo cells grow rapidly and form a confluent and polarized monolayer that exhibit morphological, hormonal and enzymatic properties common to NTB cells [Bode et al., 2006; Liu et al., 1997].

Our results indicated that ^{3}H-DG uptake by BeWo cells mainly involved a Na^{+}-independent, insulin-insensitive and low affinity (K_m = 13.4 mM), high capacity (V_{max} = 1210 nmol mg protein^{-1} 6 min^{-1}) transporter, that most probably corresponded to GLUT1 (**publication E**), which is the most functional glucose carrier present in the human STB [Baumann et al., 2002] (please see Introduction, section 2.2.4.).

To investigate the effect of oxidative stress upon the transport of glucose, BeWo cells were exposed to *tert*-butylhydroperoxide (TBHP) for 24h, which increased the intracellular levels of oxidative stress biomarkers (MDA, carbonyl groups and GSSG) without compromising cellular viability (**publication F**). Similar oxidative modifications have been found in blood of GDM women [Biri et al., 2006; Coughlan et al., 2004; Gelisgen et al., 2011; Toescu et al., 2004].

TBHP-induced oxidative stress reduced GLUT-mediated placental accumulation of ^{3}H-DG. This effect was not associated with changes in either GLUT1 mRNA levels or intracellular glucose metabolism (glycolysis). Moreover, it was associated with an increase in ^{3}H-DG paracellular and hence transepithelial transport (**publication F**). Paracellular transport of glucose, although poorly defined, has been recently demonstrated to occur across the human STB [Day et al., 2013].

The intracellular signaling pathways PI3K and PKC, known to regulate placental glucose transport [Lappas et al., 2012; Riley et al., 2005], did not seem to be involved in the inhibitory effect of oxidative stress upon ^{3}H-DG accumulation (**publication F**). Interestingly, Lappas and co-workers, although using a different oxidative challenge (hypoxanthine and xanthine oxidase) and placental model

(explants), also reported that nuclear factor kappa B and PI3K signaling pathways were not involved in the inhibitory effect of oxidative stress upon GLUT1-mediated transport [Lappas et al., 2012].

In experimental diabetic pregnancy, maternal supplementation with dietary antioxidants has been shown to reduce maternal oxidative stress and improve fetal health outcomes [Cederberg and Eriksson, 2005; Cederberg et al., 2001; Siman and Eriksson, 1997]. So, we decided to investigate if antioxidants were able to reverse the inhibitory effect of oxidative stress upon ^{3}H-DG accumulation in BeWo cells.

The dietary polyphenolic compounds quercetin, epigallocatechin-3-gallate (EGCG) and resveratrol totally abolished the reduction in ^{3}H-DG accumulation induced by oxidative stress, by specifically reversing the effect of TBHP upon GLUT-mediated transport (**publication F**). Interestingly, quercetin, EGCG and resveratrol have been previously found to prevent the inhibitory effect of oxidative stress upon the transport of other nutrients, more specifically folic acid [Couto et al., 2012], butyrate [Gonçalves et al., 2013] and glucose [Lappas et al., 2012]. Their effect was associated with a decrease in lipid peroxidation [Gonçalves et al., 2013] and with an increase in the expression of antioxidant responsive genes [Lappas et al., 2012].

Also in this work, the ROS scavengers N-acetyl-L-cysteine and α-tocopherol (the most active form of vitamin E) and inhibitors of ROS-generating enzymes (NADPH and xanthine oxidase) did not reverse the inhibitory effect of oxidative stress upon ^{3}H-DG accumulation, suggesting that this effect did not depend upon ROS generation (**publication F**). This can be explained by the fact that, for endogenous antioxidants such as vitamin E and glutathione (for which N-acetyl-L-cysteine is a precursor [Gallo et al., 2010]), the interaction of multiple systems is necessary to counteract oxidative processes. For instance, the antioxidant activity of vitamin E needs to be supported by the activity of GSH and vitamin C [Gallo et al., 2010].

GDM is associated with elevated maternal blood levels of glucose, insulin, leptin, inflammatory mediators and oxidative stress (see Introduction). The placenta, mainly the STB, may contribute for the appearance of these conditions since it produces a large number of proinflammatory cytokines (eg. TNF-α and IL-

6), hormones (eg. leptin) [Desoye and Hauguel-de Mouzon, 2007; Jansson et al., 2009] and ROS [Myatt and Cui, 2004] that can be released into both the maternal and fetal circulations.

The results presented in **chapter III (publications A, B, C and G)** identified which specific GDM molecular hallmarks interfered with the placental transport of FA, amino acids and LC-PUFAs. In this context, the effect of GDM-associated conditions - increased levels of glucose, insulin, leptin, proinflammatory mediators (lipopolysaccharide [LPS] and TNF-α) and oxidative stress - upon the uptake of ^{3}H-FA, ^{14}C-L-neutral amino acids (^{14}C-L-Met and ^{14}C-L-Alanine [^{14}C-L-Ala]) and ^{14}C-LC-PUFAs by NTB or BeWo cells were investigated. Additionally, the uptake of ^{14}C-L-Met was also characterized in BeWo cells.

The characteristics of FA transport were previously shown to be very similar in both NTB and BeWo cells [Keating et al., 2007; Keating et al., 2006; Keating and Martel], reinforcing the suitability of both cell models to study the placental transport of FA. Concerning the characterization of ^{14}C-L-Met uptake in BeWo cells, we found it to be similar to uptake by NTB cells. Both processes showed similar time-dependence and kinetics (with an affinity in the micromolar range) and were mainly Na^+-independent and system L (more specifically LAT1 isoform)-mediated, (**publication B**). Although system L was the major transporter responsible for ^{14}C-L-Met uptake in BeWo cells (± 50%) it did not account for the entire uptake. So, a non-system L-mediated transporter also appeared to participate in this process, which most probably corresponds to system y^+. In fact, besides transporting cationic amino acids (eg. L-Lys and L-arginine), system y^+ has also been demonstrated to be involved in the transport of large neutral amino acids [Battaglia and Regnault, 2001; Jansson et al., 2001; Martin-Venegas et al., 2009]. At least 3 isoforms of system y^+ are expressed in the human STB: CAT1, CAT2B and CAT4 [Carter, 2012].

The involvement of system y^+ in ^{14}C-L-Met by Bewo cells was strongly suggested by the *N*-ethylmaleimide (NEM) and L-Lys-sensitivity of non-system L-mediated uptake (NEM and L-Lys are inhibitors of system y^+-mediated transport [Deves et al., 1993]) and by the similar affinity of non-system L-mediated uptake (K_m ± 142 μM) with that of CAT1- and CAT2B-mediated uptake (K_m ± 100–400 μM) [Casanello et al., 2009; Sobrevia and Gonzalez, 2009] (**publication G**).

To study the effect of GDM-associated conditions upon ^{3}H-FA, ^{14}C-L-Met, ^{14}C-AA and ^{14}C-DHA placental uptake, BeWo or NTB cells were short- (26 min - 4h) or long-term (24-72h) exposed to those conditions.

Our results showed that short (4h) and particularly long-term (24h) hyperleptinemia decreased ^{3}H-FA uptake, and that short-term (4h) exposure to LPS (in concentrations [1-10 μg/ml] known to induce IL-6 and TNF-α secretion by trophoblasts [Torricelli et al., 2009]) or to high levels of TNF-α (300 ng/l) itself reduced ^{3}H-FA uptake in BeWo cells. On the other hand, hyperglycemia and hyperinsulinemia were devoid of effect upon ^{3}H-FA uptake (**publication A**).

Leptin has important placental functions including regulation of nutrient metabolism and trophoblast proliferation, invasion and angiogenesis [Mouzaki et al., 2012; von Versen-Hoynck et al., 2009; White et al., 2004], suggesting that this hormone may affect fetal growth [D'Ippolito et al., 2012]. The functions attributed to leptin depend upon its binding to specific receptors, which have been localized in the human STB [von Versen-Hoynck et al., 2009], resulting in activation of the following signal transduction pathways: JAK (janus kinases)/STAT (signal transducers and activators of transcription), PI3K, protein kinases (PK) A and C, and mitogen-activated protein kinases (MAPK) such as extracellular-signal-regulated-kinase (ERK), c-Jun-N-terminal kinase (JNK) and p38 MAPK [Fruhbeck, 2006; von Versen-Hoynck et al., 2009]. However, further experiments searching for the intracellular pathways involved in the inhibitory effect of long-term leptin upon ^{3}H-FA uptake in BeWo cells, showed that this effect seemed to be independent of JAK/STAT, PI3K, PKA, PKC and MAPK signaling pathways (**publication A**).

Concerning the effect of GDM conditions upon the uptake of the neutral amino acids ^{14}C-L-Met and ^{14}C-L-Ala in BeWo cells, we observed that: a) oxidative stress (TBHP; 24h) reduced the uptake of ^{14}C-L-Ala mediated by system A (**publication G**), and b) long-term hyperleptinemia, hyperglycemia (48-72h) and oxidative stress (TBHP; 24h) decreased, and hyperinsulinemia and high levels of LPS (1-50 μg/ml) and TNF-α (100-1000 ng/l) did not substantially alter, ^{14}C-L-Met uptake (**publications B and G**). Further experiments aimed at characterizing the inhibitory effect of hyperleptinemia, hyperglycemia and oxidative stress upon ^{14}C-L-Met and ^{14}C-L-Ala uptake revealed that: a) hyperglycemia and hyperleptinemia

appeared to inhibit system L-mediated ^{14}C-L-Met uptake, whereas oxidative stress appeared to inhibit system y^+-mediated ^{14}C-L-Met uptake (**publication B and G**); b) the effect of hyperglycemia upon ^{14}C-L-Met uptake seemed to be mTOR-independent, whereas the effect of hyperleptinemia upon ^{14}C-L-Met uptake seemed to be PI3K-, ERK- and p38 MAPK-dependent (**publication B**); and c) the inhibitory effect of oxidative stress upon ^{14}C-L-Ala uptake seemed to be PI3K- and PKC-dependent and was completely prevented by the polyphenolic compound quercetin (**publication G**).

Maternal intake of quercetin, one of the most abundant polyphenols found in the human diet [Aherne and O'Brien, 2002], during gestation has been reported to decrease DNA oxidation in offspring tissues, thereby decreasing their susceptibility to develop oxidative stress-related diseases latter in life [Vanhees et al., 2013]. So, we suggest that the protective role of quercetin against oxidative stress-induced inhibition of L-Ala placental transport, observed by us, is probably related with its antioxidant capacity (**publication G**).

We also studied the modulation of placental uptake of ^{14}C-AA and ^{14}C-DHA by GDM-associated conditions (**publication C**). Our results showed that, in NTB cells, short-term (26 min) exposure to high levels of TNF-α (100 ng/l) increased both ^{14}C-AA and ^{14}C-DHA uptake, whereas short-term hyperinsulinemia increased ^{14}C-DHA uptake. Hyperglycemia and hyperleptinemia were devoid of effect upon ^{14}C-AA and ^{14}C-DHA uptake (**publication C**).

Nuclear receptors such as peroxisome proliferator-activated receptors, retinoid X receptors and liver X receptors (LXR) are key regulators of placental fatty acids transport and metabolism [Duttaroy, 2009]. Since activation of LXR has been shown to increase ACSL-mediated transport of LC-PUFAs in human trophoblast [Weedon-Fekjaer et al., 2010] and inducers of proinflammatory cytokines production (such as TNF-α) [Aye et al., 2012] and insulin [Chen et al., 2004] were shown to activate LXR, we suggest that the effect of hyperinsulinemia and of high levels of TNF-α upon ^{14}C-AA and ^{14}C-DHA in NTB cells uptake may depend on LXR activation.

Altogether, the results from **chapter III** suggest that GDM-associated conditions, in particular hyperleptinemia, high inflammatory status and oxidative stress, may act as regulators of placental nutrient transport and thus of fetal

growth. On the other hand, both hyperglycemia and hyperinsulinemia do not seem to have a great impact upon the placental transport of nutrients. These results are particularly interesting considering that leptin [Bouret et al., 2004; Mouzaki et al., 2012], proinflammatory mediators [Mouzaki et al., 2012] and oxidative stress [Giussani et al., 2012; Thompson and Al-Hasan, 2012] have been reported to be important players in fetal programming.

An additional point to discuss is the contrasting effect of some GDM-associated conditions upon the transport of L-Met, FA and LC-PUFAs (**chapter III**) and the transport of these nutrients in DTB in comparison with NTB cells (**chapter I**) (Table 1). This observation implies that the effect of GDM upon the placental transport of nutrients cannot be attributed to a single GDM condition, but rather to a complex interaction between multiple conditions.

Table 1. Effect of gestational diabetes (GDM) and its associated conditions upon the placental uptake of nutrients

	Placental uptake of nutrients					
	Folic acid	**L-Met**	**L-Ala**	**DHA**	**AA**	**Glucose**
Hyperglycemia	=	↓		=	=	
Hyperinsulinemia	=	=		↑	=	
Hyperleptinemia	↓	↓		=	=	
Inflammation (TNF-α)	↓	=		↑	↑	
Inflammation (LPS)	↓	=		=	=	
Oxidative Stress		↓	↓	=	=	↓
GDM	=	=		↓	↓	

AA: arachidonic acid; DHA: docosahexaenoic acid; FA: folic acid; L-Met: L-methionine; L-Ala: L-Alanine; ↓: decrease in uptake; ↑: increase in uptake; =: uptake not altered.

As a general conclusion of this study, we can say that GDM induces alterations in placental development and transport of nutrients which may ultimately contribute to the fetal and postnatal adverse health outcomes associated with GDM. Because these nutrients are involved in the metabolism of methyl groups necessary for epigenetic regulation, we can speculate that these alterations may eventually contribute for the fetal programming effects associated with this

disease, suggesting a close link between GDM, placental function and fetal programming.

The final conclusions of this study are listed below.

With respect to the placental transport of nutrients in normal and GDM pregnancies:

1) Folic acid (FA) transport in human cytotrophoblasts isolated from normal and GDM pregnancies (NTB and DTB cells, respectively) was quantitatively similar, although in DTB cells it depended more on the activity of PCFT

2) L-methionine (L-Met) transport by NTB and DTB cells was mainly mediated by system L

3) In NTB and DTB cells, L-Met transport was quantitatively similar although it involved the interplay of different transporters: system L was involved in L-Met transport in NTB and DTB cells, but LAT2 and systems A and b^{0+} were capable of transporting L-Met only in DTB cells, and system $y^{+}L$ seemed to be more active in DTB compared with NTB cells

4) LC-PUFAs transport by NTB cells involved both a protein-mediated mechanism (dependent upon long-chain acyl-CoA synthetases - ACSL) and simple diffusion

5) Transport capacity of LC-PUFAs was markedly reduced in DTB cells, which was coincident with a reduction of similar magnitude in ACSL1 gene expression

With respect to placental development in normal and GDM pregnancies:

6) DTB cells seemed to have a higher cell turnover when compared to NTB cells

With respect to assessment of GDM-associated hallmarks and their effect upon placental nutrient transport:

7) Higher levels of oxidative stress along with an increased antioxidant capacity were present in T1D placentas, compared to normal and GDM placentas. Oxidative stress and antioxidants levels were unaltered in GDM placentas

8) Oxidative stress (induced by *tert*-butylhydroperoxide) reduced GLUT-mediated ^{3}H-2-D-deoxyglucose (^{3}H-DG) cellular accumulation, but stimulated its paracellular transport

9) Hyperleptinemia and high levels of LPS and TNF-α *per se* decreased FA uptake in a human trophoblast cell line (BeWo cells)

10) Hyperleptinemia and hyperglycemia *per se* decreased system L-mediated L-Met uptake in BeWo cells

11) Oxidative stress (induced by *tert*-butylhydroperoxide) reduced non-system L-mediated uptake of L-Met (probably via system y^{+}), and system A-mediated L-Ala uptake in BeWo cells

12) In NTB cells, high levels of TNF-α increased both AA and DHA uptake, whereas hyperinsulinemia increased only DHA uptake

Several physiological implications arise from these main conclusions:

1) In GDM pregnancies, the reduced placental uptake of LC-PUFAs and decreased ACSL1 gene expression, originating an insufficient supply of AA and DHA to the placenta and fetus, may constitute one possible mechanism underlying GDM fetal programming of neurological disorders

2) Alterations in fetal growth associated with GDM might not be explained by quantitative changes in placental transport of FA and L-Met

3) The known regulatory effect of GDM upon the placental transport of nutrients cannot not be attributed to an isolated GDM-associated condition, but rather to the interplay of multiple conditions

4) The observed increase in trophoblast proliferation and apoptosis may disturb the normal development of the placenta in GDM, and so ultimately induce perinatal and latter in life adverse health outcomes for both the mother and fetus

5) The actual GDM diagnosis, based on the new IADPSG criteria, is likely to be diagnosing with GDM, women that may not really show relevant dysglycemia or oxidative imbalance

FUTURE PERSPECTIVES

We believe that this work contributed for a better knowledge on the impact of GDM upon the placental transport of nutrients and placental development. However, it also showed that new aspects should be investigated in more detail, namely:

a) the modulation of placental transport of cationic (eg. L-arginine) and neutral (eg. L-serine and L-histidine) amino acids by GDM and its associated conditions. These amino acids are substrates of LAT2 and y^+LAT1 transporters and of systems A, y^+ and $b^{0,+}$ [Cleal and Lewis, 2008], which are altered in GDM,

b) the effect of GDM-associated conditions upon the invasive, angiogenic and migration capacities of extravillous trophoblasts,

c) the search for epigenetic modifications (DNA methylation) in placental FA, L-Met and LC-PUFAs transporter genes in GDM

d) the impact of maternal AA and DHA supplementation during the perigestational period (which extends from conception throughout pregnancy till the end of lactation) in the metabolic and neurologic phenotype of offsprings born from GDM women.

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SUMMARY

SUMMARY

Gestational diabetes mellitus (GDM) is the most prevalent metabolic disorder diagnosed in pregnant women. This disease increases the risk of adverse perinatal outcomes and later in life metabolic and neurological diseases for the newborn. However, the cellular and molecular mechanisms underlying these pathological and programming effects of GDM are still largely unexplored.

The human placenta is the main interface between the maternal and fetal blood circulations, being responsible - at the level of the syncytiotrophoblast epithelium - for the maternal-to-fetal transfer of nutrients essential for fetal growth and development. In this context, we hypothesized that changes in placental transport function and development may be present in GDM pregnancies.

So, our main aim was to investigate the effect of GDM and its associated hallmarks (hyperglycemia, hyperinsulinemia, hyperleptinemia and elevated levels of inflammation and oxidative stress) upon the placental transport of folic acid (FA), neutral amino acids (L-methionine [L-Met] and L-alanine [L-Ala]), long-chain polyunsaturated fatty acids (arachidonic [AA] and docosahexaenoic [DHA] acids) and glucose, and upon placental development. Additionaly, we also aimed to assess oxidative stress status in GDM placentas.

Our results led us to conclude that: a) FA and L-Met uptake by human trophoblasts isolated from both normal and GDM pregnancies (NTB and DTB cells, respectively) was quantitatively similar, although it involved the interplay of distinct transporters; b) AA and DHA uptake (which was both mediated by simple diffusion and by transporter proteins) was markedly reduced in DTB cells, coincident with a similar reduction of long-chain acyl-CoA synthetase (ACSL) 1 gene expression; and c) proliferation and apoptosis was higher in DTB than in NTB cells, suggesting a higher cell turnover.

With respect to GDM-associated conditions, we verified that: a) in a human trophoblast cell line (BeWo cells) cells, hyperleptinemia and high levels of the inflammatory mediators lipopolysaccharide and tumor necrosis factor-alpha (TNF-α) decreased FA uptake, and hyperleptinemia and hyperglycemia decreased system L-mediated L-Met uptake; b) in NTB cells high levels of TNF-α increased both AA and DHA uptake and hyperinsulinemia increased DHA uptake; c) placental oxidative stress and antioxidant levels were found to be increased in type 1 diabetic pregnant women but unaltered in GDM women; and d) oxidative stress

(induced by *tert*-butylhydroperoxide) reduced the uptake of L-Met, most probably mediated by system y^+, reduced system A-mediated uptake of L-Ala, and reduced facilitative glucose transporters (GLUT)-mediated 2-D-deoxyglucose (DG) uptake.

In conclusion, GDM disturbs placental transport, and consequently fetal supply, of nutrients and it also alters trophoblast development. These effects eventually contribute to the fetal and post-natal adverse health outcomes associated with GDM.

RESUMO

RESUMO

A diabetes *mellitus* gestacional (DMG) é a doença metabólica mais comum diagnosticada em mulheres grávidas. Esta doença aumenta o risco de morbilidade perinatal bem como de doenças metabólicas e neurológicas a longo prazo no recém-nascido. Contudo, os mecanismos moleculares e celulares responsáveis por este efeito patológico e programador da DMG estão ainda muito pouco explorados.

A placenta humana constitui a principal ligação entre a mãe e o feto, sendo responsável – ao nível do epitélio do sincíciotrofoblasto – pelo transporte materno-fetal de nutrientes necessários ao crescimento e ao desenvolvimento do feto. Neste contexto, levantamos a hipótese de que a DMG induz alterações na função transportadora e no desenvolvimento da placenta.

Como tal, o nosso principal objetivo foi investigar o efeito da DMG e das condições a ela associadas (hiperglicemia, hiperinsulinemia, hiperleptinemia e níveis elevados de inflamação e stresse oxidativo) sobre o transporte placentário de ácido fólico (AF), aminoácidos (L-metionina [L-Met] e L-alanina [L-Ala]), ácidos gordos polinsaturados de cadeia longa (ácido araquidónico [AA] e docosahexaenóico [ADH]) e glicose, bem como sobre o desenvolvimento da placenta. Adicionalmente, foi também nosso objetivo avaliar o estado redox placentário na DMG.

Os nossos resultados levaram-nos a concluir que: a) a captação de AF e L-Met em trofoblastos humanos provenientes de gravidezes normais e com diabetes gestacional (células NTB e DTB, respetivamente) foi quantitativamente semelhante, apesar de estarem envolvidos mecanismos de transporte distintos; b) a captação de AA e ADH (que envolveu difusão simples mas também proteínas transportadoras) é marcadamente inferior nas células DTB, sendo acompanhado por uma redução da mesma ordem de grandeza da expressão génica da sintétase de acil-CoA de cadeia longa (ACSL) 1; e c) a proliferação e apoptose foi maior nas células DTB comparativamente às NTB, sugerindo uma maior velocidade renovação celular nas primeiras.

Relativamente às condições da DMG, verificamos que: a) numa linha celular de trofoblastos humanos (células BeWo) a hiperleptinemia e níveis elevados dos mediadores inflamatórios lipopolissacarídeo e fator de necrose tumoral-alfa (TNF-α) reduziram a captação de AF, e a hiperleptinemia e a hiperglicemia reduziram a captação de L-Met mediada pelo sistema L; b) em células NTB, níveis elevados de

TNF-α aumentaram a captação de AA e ADH e a hiperinsulinemia aumentou a captação de ADH, c) os níveis de stresse oxidativo e defesas antioxidantes estavam aumentados nas placentas provenientes de mulheres grávidas com diabetes tipo 1, mas não estavam alterados nas placentas provenientes de mulheres diabéticas gestacionais, e d) o stresse oxidativo (induzido pelo *tert*-butilhidroperóxido) reduziu a captação de L-Met mediada pelo sistema y^+, a de L-Ala mediado pelo sistema A, e a de 2-D-desoxiglicose (DG) mediada pelos transportadores facilitativos de glicose (GLUT).

Em conclusão, a DMG altera o transporte placentário, e consequentemente a disponibilidade fetal, de nutrientes bem como o desenvolvimento dos trofoblastos. Estas alterações podem eventualmente contribuir para os efeitos adversos da DMG sobre o feto e sobre o recém-nascido.