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

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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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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**).

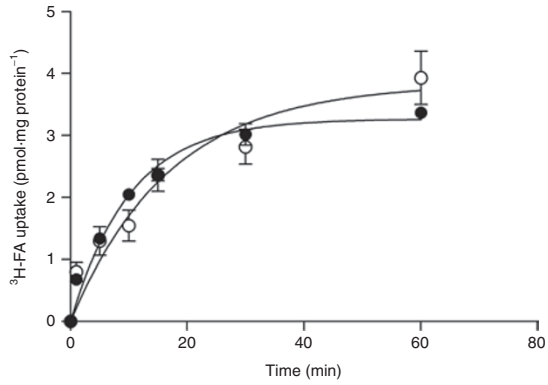

Figure 1. Time-course of ^{3}H-FA apical uptake by NTB (open circles) and DTB (solid circles) cells. Cells were incubated at pH 5.5 with 20 nmol/l ^{3}H-FA for various periods of time (n = 6–8, from three or four distinct placentas). Values shown are arithmetic means ± SEM. DTB, diabetic trophoblasts;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

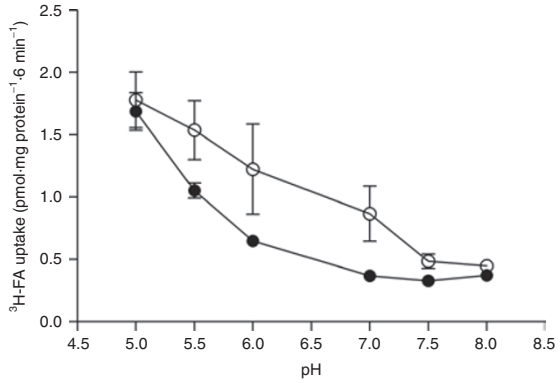

Figure 2. pH-dependence of ^{3}H-FA apical uptake by NTB (open circles) and DTB (solid circles) cells. Initial rates of uptake were determined in cells incubated with 20 nmol/l ^{3}H-FA for 6 min at extracellular pH ranging from 5.0 to 8.0. Values shown are arithmetic means ± SEM (n = 4–8, from two to 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.

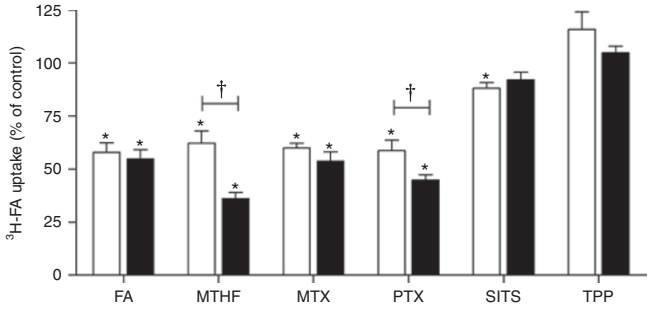

Figure 3. Effect of folic acid (FA; 100 µmol/l), 5-methyltetrahydrofolate (MTHF; 10 µmol/l), methotrexate (MTX; 10 µmol/l), pemetrexed (PTX; 20 µmol/l), 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid disodium salt hydrate (SITS; 500 µmol/l), and thiamine pyrophosphate (TPP; 100 µmol/l) on ^{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).

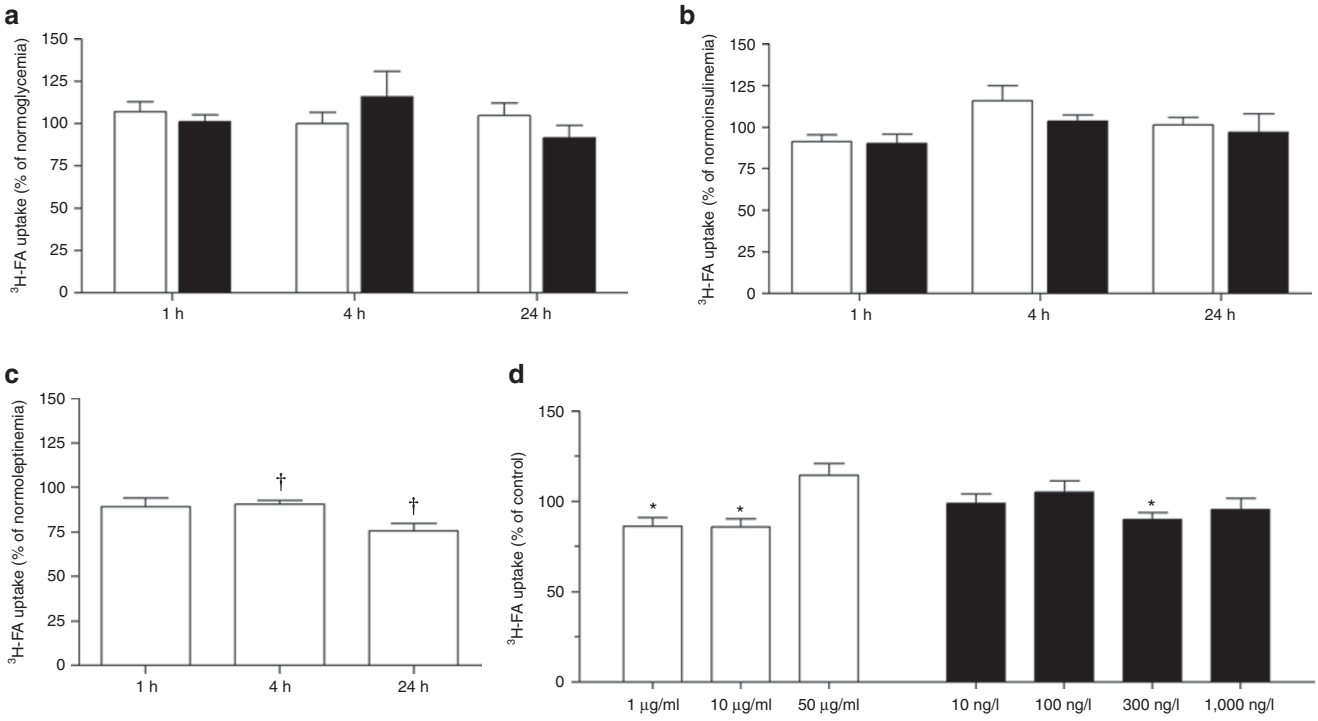

Figure 4. Effect of GDM-associated conditions on ^{3}H-FA apical uptake by BeWo cells. Cells were exposed to (**a**) D-glucose at 10 or 30 mmol/l (white and black bars respectively; $n = 8–9$) or 5.6 mmol/l (supplemented with mannitol up to 10 or 30 mmol/l for isosmotic control, corresponding to normoglycemia (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.

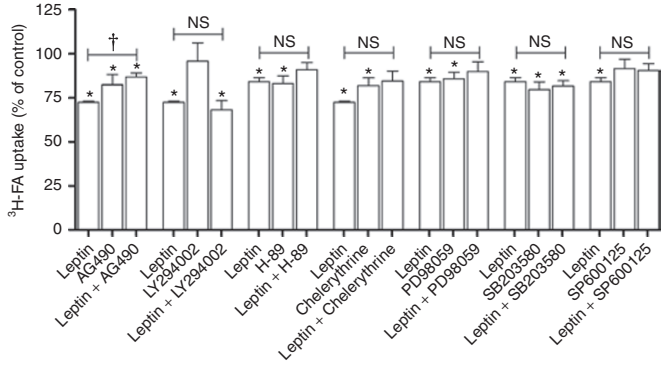

Figure 5. Effect of inhibitors of signaling pathways on the apical uptake of ^{3}H-FA and on leptin-induced inhibition of ^{3}H-FA uptake by BeWo cells. Initial rates of uptake were determined in cells incubated for 6 min with 50 nmol/l ^{3}H-FA after treatment for 24 h with 100 ng/ml leptin, 5 µmol/l 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).

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

$A(t)$ 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.

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