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Progesterone Inhibits Folic Acid Transport in Human Trophoblasts

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Abstract The aim of this work was to test the putative involvement of members of the ABC superfamily of transporters on folic acid (FA) cellular homeostasis in the human placenta. [^{3}H]FA uptake and efflux in BeWo cells were unaffected or hardly affected by multidrug resistance 1 (MDR1) inhibition (with verapamil), multidrug resistance protein (MRP) inhibition (with probenecid) or breast cancer resistance protein (BCRP) inhibition (with fumitremorgin C). However, [^{3}H]FA uptake and efflux were inhibited by progesterone (200 μM). An inhibitory effect of progesterone upon [^{3}H]FA uptake and efflux was also observed in human cytotrophoblasts. Moreover, verapamil and ß-estradiol also reduced [^{3}H]FA efflux in these cells. Inhibition of [^{3}H]FA uptake in BeWo cells by progesterone seemed to be very specific since other tested steroids (β-estradiol, corticosterone, testosterone, aldosterone, estrone and pregnanediol) were devoid of effect. However, efflux was also inhibited by β-estradiol and corticosterone and stimulated by estrone. Moreover, the effect of progesterone upon the uptake of [^{3}H]FA by BeWo cells was concentration-dependent (IC_{50} = 65 [range 9–448] μM) and seems to involve competitive inhibition. Also, progesterone (1–400 μM) did not affect either [^{3}H]FA uptake or efflux at an external acidic pH. Finally, inhibition of [^{3}H]FA uptake by progesterone was unaffected by either 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid (SITS), a known inhibitor of the reduced folate carrier (RFC), or an anti-RFC antibody. These results suggest that progesterone inhibits RFC. In conclusion, our results show that progesterone, a sterol produced by the placenta, inhibits both FA uptake and efflux in BeWo cells and primary cultured human trophoblasts.

Keywords Folic acid homeostasis · Placenta · Progesterone · Steroid · ABC transporter · Reduced folate carrier

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Introduction

Folic acid (vitamin B_9, FA) is an essential micronutrient for normal cellular functions, growth and development. Reduced derivatives of this water soluble vitamin play a key role in one-carbon transfer reactions in the *de novo* biosynthesis of purines and thymidylate (Herbert, 1999; Worthington-Roberts, 1999; Lucock, 2000). As such, dividing cells have an absolute requirement for reduced folates in order to properly perform DNA replication and mitosis (Herbert, 1999; Worthington-Roberts, 1999; Lucock, 2000). Indeed, disruption of one-carbon transfer reactions in the folate metabolic pathway is the pharmacological basis for the antitumor activity of folate antagonists (i.e., antifolates) such as methotrexate (McGuire, 2003).

FA is particularly important during pregnancy, when optimal placental uptake of FA from the maternal circulation is critical for normal development and growth of the placenta and for an adequate supply of FA to the

developing fetus. The importance of FA during pregnancy is supported by the fact that maternal FA deficiency is associated with various complications such as low birth weight, increased risk of spontaneous abortion and neural tube defects (e.g., spina bifida and anencephaly) and by accumulating evidence that supplementation with this vitamin during the periconceptional period of pregnancy can reduce the incidence of low birth weight newborns and neural tube defects (Herbert, 1999; Worthington-Roberts, 1999; Lucock, 2000; Picciano, 2003).

Maternal-to-fetal transfer of FA occurs at the level of the polarized epithelial cell-like syncytiotrophoblast (the outermost layer of the placental villi) and depends on the transcellular transport of FA across these cells. Using the BeWo human choriocarcinoma cell line as a placental syncytiotrophoblast model, our group showed that, at physiological pH, cellular uptake of FA from the maternal circulation (which occurs at the apical membrane of syncytiotrophoblasts) seems to involve two distinct mechanisms: folate receptor-α and the reduced folate carrier (RFC) (Keating et al., 2006). Folate receptor-α is a glycosylphosphatidylinositol-anchored protein located at the apical membrane of these cells, in direct contact with the maternal blood, and binds FA with high affinity (in the nanomolar range) (Antony, 1996; Matherly & Goldman, 2003). RFC (SLC19A1) is a membrane transporter driven by the transmembrane H^+ gradient (Sirotnak & Tolner, 1999; Matherly & Goldman, 2003). Both these transporters are present in BeWo cells (Keating et al., 2007) and human placenta (Sirotnak & Tolner, 1999; Matherly & Goldman, 2003), and they seem to be involved in FA uptake by primary cultured human trophoblasts (Keating et al., 2005).

However, cellular FA pools are not only controlled by the above FA uptake systems and by the activity of folylpoly-γ-glutamate synthetase (an enzyme that catalyzes the addition of several equivalents of L-glutamate to the γ-carboxyl group in the side chain of folate cofactors following their uptake) but also depend on efflux transporters, which are able to remove FA from the cells. Among these, several members of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of efflux transporters are known to accept FA and/or antifolates such as methotrexate as substrates (Matherly & Goldman, 2003), namely, members of the multidrug resistance protein (MRP) family (ABCC1-5 or MRP1-5) (Zeng et al., 2001; Chen et al., 2002; Assaraf et al., 2003; Hooijberg et al., 2003) and the breast cancer resistance protein (BCRP or ABCG2) (Chen et al., 2003; Ifergan et al., 2004). These ATP-dependent transporters are all known to be present at the placental level (Young, Allen & Audus, 2003). However, the influence of these transporters on the intracellular pools of FA in the syncytiotrophoblast, and consequently on maternal-to-fetal FA transport, remains to be elucidated.

The aim of this work was to test the putative involvement of transporters belonging to the ABC superfamily of transporters on FA transport by human syncytiotrophoblasts. For this purpose, we tested the effect of selective inhibitors of these transporters on both [^{3}H]FA influx and efflux in two cell culture models of syncytiotrophoblasts: BeWo cells and primary cultured human trophoblasts. We focused our attention on MRP (ABCC) family members and on BCRP (ABCG2). Additionally, we investigated the putative involvement of multidrug resistance 1 or P-glycoprotein (MDR1, ABCB1), the best known of all transporters involved in the phenomenon of multidrug resistance (Borst & Oude Elfering, 2002; Fromm, 2004). Although it is not known wether MDR1 transports FA, this transporter is present in human placenta and BeWo cells (Sugawara et al., 1997; Nakamura et al., 1997; Ushigome et al., 2000; Utoguchi et al., 2000).

Materials and Methods

BeWo Cell Culture

The BeWo cell line was obtained from the American Type Culture Collection (ATCC CCL-98, Barcelone, Spain) and used between passage numbers 11 and 40. The cells were maintained in a humidified atmosphere of 5% CO_2-95% air and 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 2–3 days, and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-ethylenediaminetetraacetic acid [EDTA], 5 min, 37°C), split 1:2–1:3 and subcultured in plastic culture dishes (21 cm^2, ∅ 60 mm; TPP. Trasadingen, Switzerland). For the experiments, BeWo cells were seeded on collagen-coated 24-well plastic cell culture clusters (2 cm^2, ∅ 16 mm; TPP). After 3–5 days in culture (90–100% confluence), the cells were used in transport experiments. Each square centimeter contained about 60 μg cell protein.

Primary Cultures of Human Trophoblasts

Human placentas were obtained at the Department of Obstetrics, Hospital de São João (Porto, Portugal), from uncomplicated term pregnancies (37–40 weeks, $n = 5$) within half an hour after spontaneous delivery or elective cesarean-section. Collection and processing of human placentas were approved by the hospital ethical committee.

Cytotrophoblasts were isolated and cultured using a modification of the technique described by Kliman et al.

(1986). Briefly, fetal membranes and maternal decidua were removed, and villous tissue was cut and scraped from blood vessels. Blood was removed by extensive washing with 2 l of saline. Tissue was then digested in Hanks balanced salt solution (HBSS) containing 0.15% trypsin, 0.02% DNAse I and 1% antibiotic/antimycotic. Supernatants were pooled and filtered through a sterile stainless wire mesh sieve with a 100-μm pore. Cells were pelleted at 1,800 rpm for 10 min at room temperature and resuspended in Dulbecco's modified Eagle medium (DMEM, with 1% antibiotic/antimycotic). Suspensions were layered on top of a freshly prepared 70% to 5% Percoll® (Sigma, St. Louis, MO, USA) density discontinuous gradient and centrifuged at 1,200 x *g* for 20 min at room temperature. Cells banding at densities between 1.049 and 1.062 g/ml Percoll®, corresponding to villous trophoblasts, were collected and centrifuged at 1,800 rpm for 10 min at room temperature. The pellet of trophoblasts was resuspended in DMEM/F-12 (containing 10% fetal calf serum and 1% antibiotic/antimycotic), and trophoblasts were seeded on 24-well plastic cell culture clusters (2 cm^2, ∅ 16 mm; TPP) at a density of 2.5 to 5×10^5 cells/cm^2. After 3–4 h in culture, nonadherent cells were removed by washing with phosphate-buffered saline at 37°C. Medium was changed daily. After 72 h in culture, placental trophoblast cells aggregated to form syncytial clumps corresponding to syncytiotrophoblasts and were then used for experiments.

Transport Studies

Transport experiments were performed in buffer with the following composition (in mM): 125 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 12.5 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH, 12.5 2-(*N*-morpholino)ethanesulfonic acid (MES), 1.2 $MgSO_4$, 1.2 $CaCl_2$ and 5.6 D(+)-glucose (pH 7.5).

Influx transport experiments

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 10 or 20 nM [^{3}H]FA (except where indicated). Incubation was stopped 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. Drugs to be tested were present during both the preincubation and incubation periods.

Efflux transport experiments

Initially, the culture medium was aspirated and the cells were washed with buffer at 37°C. Then, the cell monolayers were incubated for 30 min with 0.2 ml buffer at 37°C containing 10 or 20 nM [^{3}H]FA. Incubation was stopped by removing the incubation medium and rinsing the cells with 0.5 ml ice-cold buffer. Then, efflux was measured by incubating the cells with buffer at 37°C for 20 min. At the end of this period, the medium was collected and the cells were 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 both the efflux buffer and the cells was measured by liquid scintillation counting. Drugs to be tested were present during the efflux period only.

Protein Determination

The protein content of cell monolayers was determined as described by Bradford (1976), using bovine serum albumin as standard.

Calculations and Statistics

Arithmetic means are given with standard errors of the mean (SEM), and geometric means are given with 95% confidence limits. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance, followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. Differences were considered significant at $p < 0.05$.

Materials

[^{3}H]FA ([3′,5′,7,9-[^{3}H]]FA potassium salt, specific activity 21.0 Ci/mmol; Amersham Pharmacia Biotech, Aylesbury, UK), aldosterone, antibiotic/antimycotic solution (100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B), collagen type I from rat tail, corticosterone, DMEM (high glucose), DMEM/F-12 (Nutrient Mixture F-12 Ham), DNase I (deoxyribonuclease I from bovine pancreas), 17ß-estradiol, estrone, fetal calf serum, Ham's F12 K (Nutrient Mixture F12-Ham Kaighn's modification), HEPES, indomethacin, MES hydrate, *p*-aminohypuric acid, Percoll®, pregnanediol, probenecid,

progesterone, testosterone, trypsin-EDTA solution and verapamil hydrochloride were from Sigma (St. Louis, MO, USA). Dimethylsulfoxide (DMSO), Triton X-100 and Tris (tris-[hydroxymethyl]-aminomethane hydrochloride) were from Merck (Darmstadt, Germany). Fetal calf serum, HBSS 10x and trypsin 2.5% (10x) were from GIBCO, Invitrogen (Carlsbad, CA, USA).

Fumitremorgin C was kindly donated by Dr. Susan Bates (Molecular Therapeutics Section, Medical Oncology Branch, National Cancer Institute, Bethesda, MD, USA). The anti-RFC antibody was generated as described by Chancy et al. (2000).

When the drugs to be tested were dissolved in DMSO or ethanol, the final concentration of these solvents in the buffer was 1%. Controls for these drugs were run in the presence of the solvent.

Results

Previous experiments from our group showed that both BeWo cells and primary cultured human trophoblasts remove [^{3}H]FA from the extracellular medium in a time-dependent way, uptake at pH 7.5 being linear with time up to the sixth minute of incubation and reaching a steady state after 30 min of incubation (Keating et al., 2005, 2006). Based on this information, we chose a 6-min incubation time in order to study the effect of compounds upon the uptake (inward transport) of [^{3}H]FA and a 30-min incubation time in order to study the effect of drugs upon both the uptake (inward transport) and efflux (outward transport) of [^{3}H]FA.

Temperature Dependence of [^{3}H]FA Uptake by BeWo Cells

Uptake of [^{3}H]FA by BeWo cells after a 6-min incubation time was greatly increased when cells were preincubated and incubated at 4°C (it corresponded to 385 ± 28% of uptake at 37°C, n = 5). Transporters belonging to the ABC superfamily of transporters are normally involved in the efflux of compounds from cells, and they are ATP-dependent transporters. Thus, the above observation led us to hypothesize that a transporter of the ABC superfamily might be involved in FA homeostasis in these cells.

Effect of Inhibitors of ABC Transporters on Influx of [^{3}H]FA into BeWo Cells

In a first series of experiments, we determined the influence of inhibitors of different ABC transporters upon the uptake of [^{3}H]FA by BeWo cells both after a 6-min and after a 30-min incubation period.

As can be seen in Figure 1, uptake of [^{3}H]FA by BeWo cells was not affected by verapamil, probenecid, fumitremorgin C and ß-estradiol. However, indomethacin (100 µM) decreased the 30-min [^{3}H]FA uptake by 20%. Moreover, progesterone (200 µM) was able to significantly decrease both the 6- and the 30-min uptake of [^{3}H]FA (by 25% and 40%, respectively; Fig. 1).

Effect of Inhibitors of ABC Transporters on Efflux of [^{3}H]FA from BeWo Cells

Next, we examined the influence of the same compounds upon the efflux of [^{3}H]FA from BeWo cells. With the exception of progesterone, all the compounds tested were devoid of effect. Progesterone (200 µM) was able to

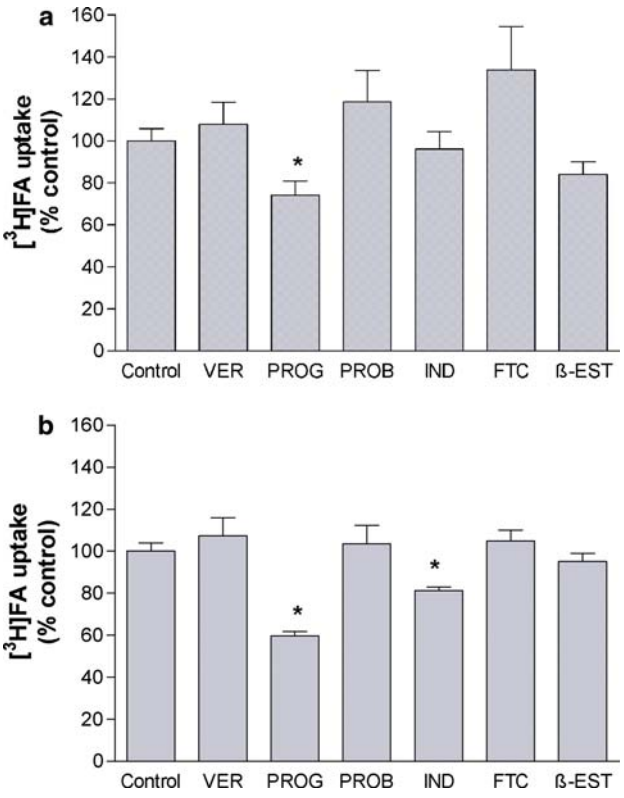

Fig. 1 Effect of various compounds upon the apical uptake of [^{3}H]FA by BeWo cells. The cell monolayers were incubated at 37°C for (**a**) 6 min or (**b**) 30 min with [^{3}H]FA (20 nM), in the absence (*Control*) or presence of verapamil (*VER*) 20 µM (n = 4–9), progesterone (*PROG*) 200 µM (n = 9–10), probenecid (*PROB*) 500 µM (n = 3–7), indomethacin (*IND*) 100 µM (n = 8–9), fumitremorgin C (*FTC*) 5 µM (n = 6–8) or 17ß-estradiol (*ß-EST*) 30 µM (n = 9–10). [^{3}H]FA uptake in control cells amounted to 0.31 ± 0.04 pmol · mg protein^{-1} (n = 16) (**a**) and 0.34 ± 0.03 pmol · mg protein (n = 9) (**b**). The results are shown as arithmetic means ± SEM. *$p < 0.05$ compared to control condition

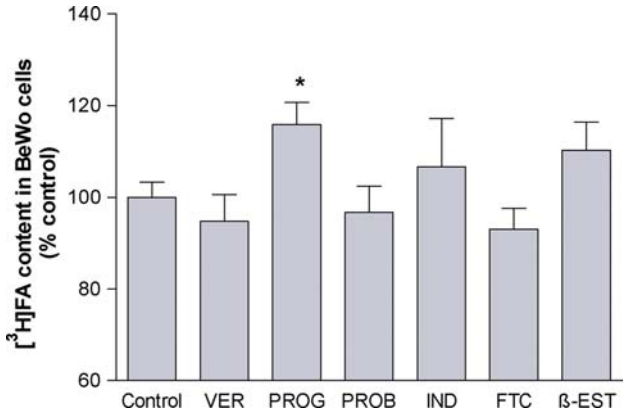

Fig. 2 Effect of various compounds on the apical efflux of [^{3}H]FA from BeWo cells. The cell monolayers were incubated at 37°C for 30 min with [^{3}H]FA (20 nM), after which efflux of [^{3}H]FA into the extracellular medium was allowed for 20 min in the absence (*Control*) or presence of verapamil (*VER*) 20 μM (n = 7), progesterone (*PROG*) 200 μM (n = 18), probenecid (*PROB*) 500 μM (n = 7), indomethacin (*IND*) 100 μM (n = 6), fumitremorgin C (*FTC*) 5 μM (n = 8) or 17ß-estradiol (*ß-EST*) 30 μM (n = 14). Shown is the [^{3}H]FA content of the cells after the efflux period, in percentage of the respective control. [^{3}H]FA uptake in control cells amounted to 0.13 ± 0.01 pmol · mg protein^{-1} (n = 15). The results are shown as arithmetic means ± SEM. *p < 0.05 compared to control condition

significantly decrease efflux of [^{3}H]FA, thus increasing the cellular content of this vitamin (Fig. 2).

Concentration Dependence of the Effect of Progesterone on [^{3}H]FA Influx and Efflux in BeWo Cells

In order to better characterize the effect of progesterone upon [^{3}H]FA uptake and efflux, we determined the concentration dependence of its effect. As shown in Figure 3a, the effect of progesterone upon [^{3}H]FA uptake was concentration-dependent; the highest concentration of progesterone tested (400 μM) reduced [^{3}H]FA uptake to 48 ± 3% of control, and its 50% inhibitory concentration (IC_{50}) was 64.9 (range 9.4–448) μM (n = 4–6). The effect of progesterone upon [^{3}H]FA efflux from BeWo cells, however, showed no concentration dependence (Fig. 4a).

Effect of Other Steroids on [^{3}H]FA Influx and Efflux in BeWo Cells

We also tested the effect of several other steroids upon [^{3}H]FA uptake and efflux from BeWo cells. Interestingly, none of the steroids tested (ß-estradiol, testosterone, corticosterone, aldosterone, estrone and pregnanediol) caused any significant alteration in [^{3}H]FA uptake (Fig. 3b). On the other hand, ß-estradiol (1 and 200 μM) and corticosterone (200 μM) inhibited [^{3}H]FA efflux from BeWo cells, and estrone (50 μM) increased it (Fig. 4b).

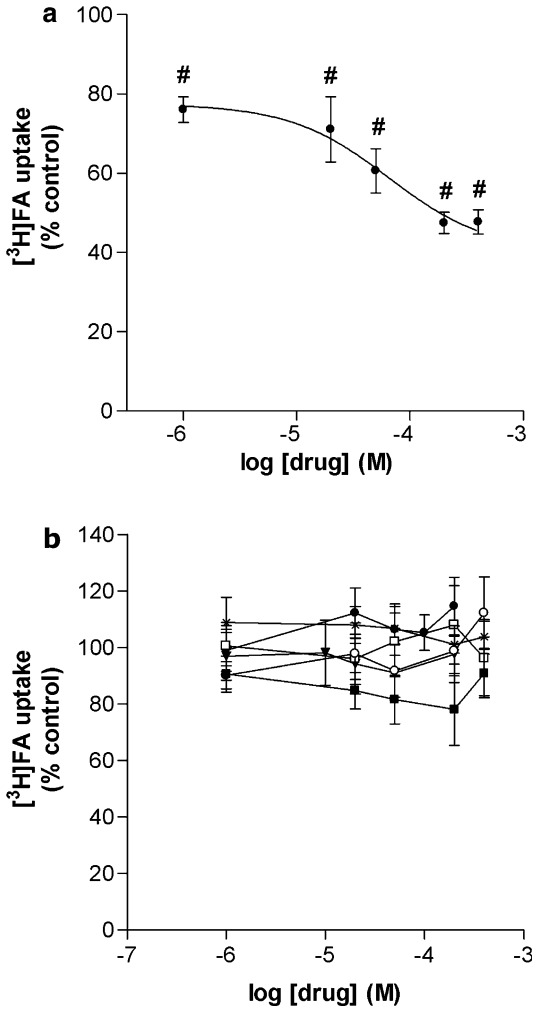

Fig. 3 Effect of several steroids on the apical uptake of [^{3}H]FA by BeWo cells. The cell monolayers were incubated at 37°C for 6 min with [^{3}H]FA (20 nM), in the absence or presence of (**a**) progesterone (*filled circles*, n = 4–6) or (**b**) 17ß-estradiol (*filled squares*, n = 6), corticosterone (*open circles*, n = 6), testosterone (*open squares*, n = 6), aldosterone (*asterisks*, n = 5–6), estrone (*filled circles*, n = 6) or pregnanediol (*filled triangles*, n = 8). [^{3}H]FA uptake in control cells amounted to 0.28 ± 0.03 pmol · mg protein^{-1} (n = 20). The results are shown as arithmetic means ± SEM. *p < 0.05 compared to control condition

Effect of Progesterone on the Kinetic Parameters of [^{3}H]FA Influx in BeWo Cells

Under control conditions, initial rates of [^{3}H]FA apical influx were saturable (Fig. 5), with an apparent Michaelis-Menten constant, K_m, of 19.6 μM (95% confidence interval 8.6–30.6) and a maximal transport rate, V_{max}, of 281 ± 35 pmol · mg protein^{-1} · 6 min^{-1}. In the presence of progesterone (200 μM), a significant increase of K_m was observed (35.0 μM range 25.6–44.5) but the V_{max} was not significantly changed (282 ± 20 pmol · mg protein^{-1} · 6 min^{-1}) (Fig. 5).

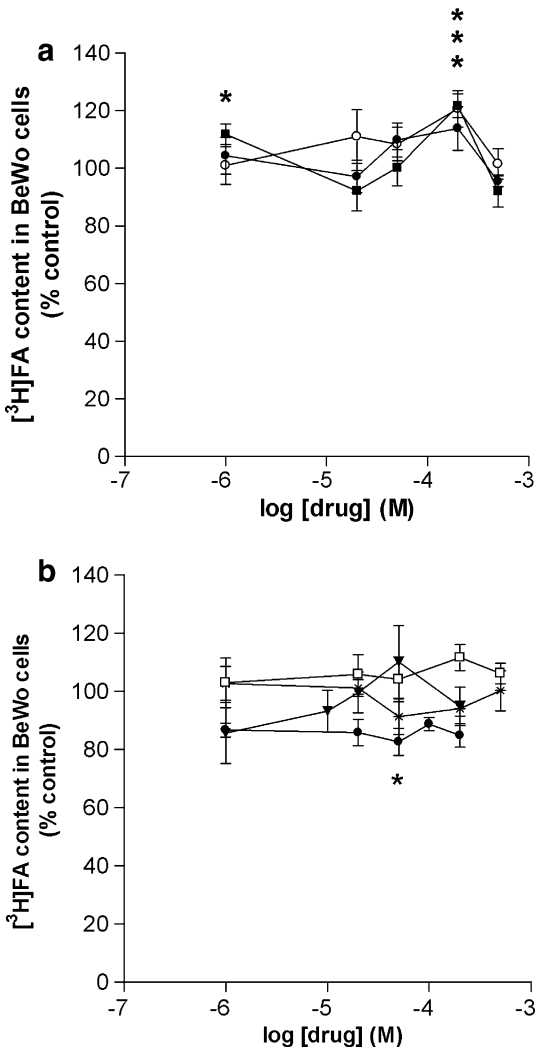

Fig. 4 Effect of several steroids on the apical efflux of [^{3}H]FA from BeWo cells. The cell monolayers were incubated at 37°C for 30 min with [^{3}H]FA (20 nM), after which efflux of [^{3}H]FA into the extracellular medium was allowed for 20 min in the absence or presence of (**a**) progesterone (*filled circles*, n = 5–6), 17ß-estradiol (*filled squares*, n = 6) or corticosterone (*open circles*, n = 6) or (**b**) testosterone (*open squares*, n = 6), aldosterone (*asterisks*, n = 6–8), estrone (*filled circles*, n = 6–8) or pregnanediol (*filled triangles*, n = 6–8). Shown is the [^{3}H]FA content of the cells after the efflux period. [^{3}H]FA uptake in control cells amounted to 0.11 ± 0.01 pmol · mg protein^{-1} (n = 19). The results are shown as arithmetic means ± SEM. *$p < 0.05$ compared to control condition

Effect of RFC Inhibition on Progesterone-Induced Inhibition of [^{3}H]FA Influx in BeWo Cells

In order to further investigate the nature of the inhibitory effect of progesterone, we tested the possibility of RFC inhibition by this steroid. For this purpose, we tested the influence of RFC inhibition (using 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid [SITS] or an anti-RFC antibody) upon the progesterone-induced reduction of [^{3}H]FA influx in BeWo cells.

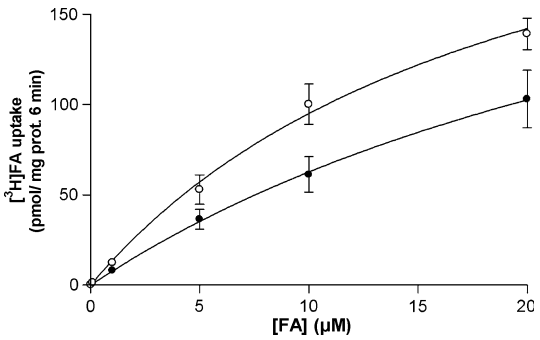

Fig. 5 Effect of progesterone upon the kinetic parameters of the apical uptake of [^{3}H]FA by BeWo cells. The cell monolayers were incubated at 37°C for 6 min with increasing concentrations of [^{3}H]FA in the absence (*open circles*) or presence (*closed circles*) of progesterone (200 µM n = 5–9). The results are shown as arithmetic means ± SEM

Interestingly, we observed that the inhibitory effect of progesterone (200 µM) upon the 6-min influx of [^{3}H]FA (69.3 ± 3.5% of control, n = 17) was not significantly changed in the presence of SITS (500 µM) (64.5 ± 4.7% of control, n = 6). SITS alone (500 µM) reduced [^{3}H]FA uptake to 79.7 ± 4.1% of control (n = 6). Similarly, the inhibitory effect of progesterone was also not affected by the presence of an anti-RFC antibody (20 µg/ml) (62.3 ± 7.4% of control, n = 6). Alone, the anti-RFC antibody reduced [^{3}H]FA influx to 61.8 ± 4.0% of control (n = 6). The amount of ^{3}H-FA present under control conditions was 0.24 ± 0.04 pmol · mg protein^{-1} (n = 17).

Effect of Progesterone on [^{3}H]FA Influx and Efflux at Low pH in BeWo Cells

We also tested the effect of progesterone at a low medium pH (5.5). Interestingly enough, progesterone (1–400 µM) was devoid of effect both on [^{3}H]FA influx and on efflux under these conditions (*results not shown*).

Effect of Inhibitors of Organic Anion Transporters on Influx and Efflux of [^{3}H]FA in BeWo Cells

We also tested the effect of *p*-aminohippuric acid (200 µM), a well-known substrate/inhibitor of the organic anion family of transporters, on both the uptake and efflux of [^{3}H]FA from BeWo cell. This compound did not change either the uptake or the efflux of [^{3}H]FA from these cells ([^{3}H]FA content in the cells in the presence of this compound corresponded to 95.2 ± 7.7% and 107.0 ± 6.5% of control, respectively; n = 5–6).

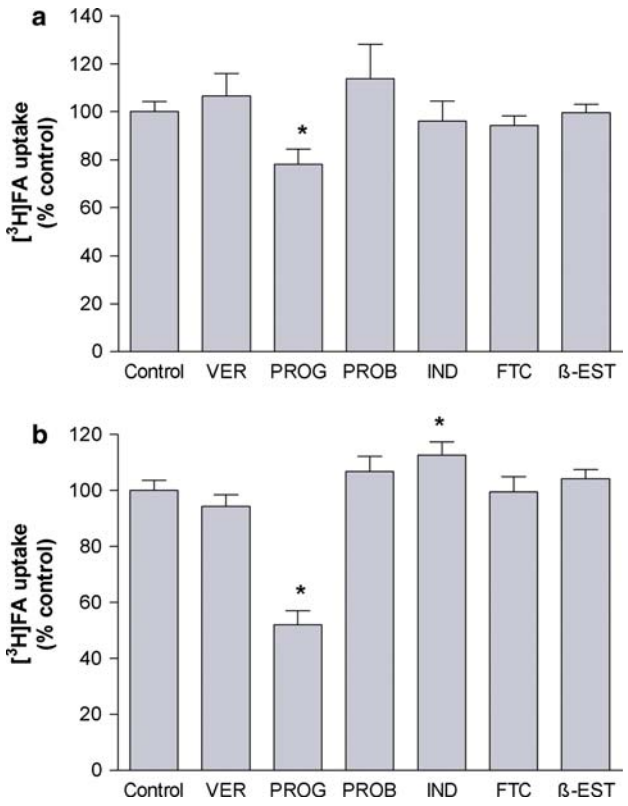

Fig. 6 Effect of various compounds upon the apical uptake of [^{3}H]FA by primary cultured human trophoblasts. The cell monolayers were incubated at 37°C for (**a**) 6 min or (**b**) 30 min with [^{3}H]FA (10 nM) in the absence (*Control*) or presence of verapamil (*VER*) 20 μM (n = 8), progesterone (*PROG*) 200 μM (n = 8–16), probenecid (*PROB*) 500 μM (n = 7–8), indomethacin (*IND*) 100 μM (n = 8), fumitremorgin C (*FTC*) 5 μM (n = 8) or 17ß-estradiol (*ß-EST*) 30 μM (n = 8–12). [^{3}H]FA uptake in control cells amounted to 0.36 ± 0.06 pmol · mg protein^{-1} (n = 8) (**a**) and 0.47 ± 0.04 pmol · mg protein^{-1} (n = 8) (**b**). The results are shown as arithmetic means ± SEM. *$p < 0.05$ compared to control condition

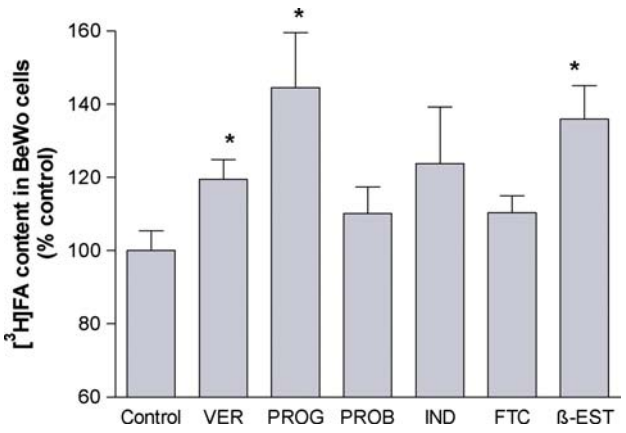

Fig. 7 Effect of various compounds on the apical efflux of [^{3}H]FA from primary cultured human trophoblasts. The cell monolayers were incubated at 37°C for 30 min with [^{3}H]FA (10 nM), after which efflux of [^{3}H]FA into the extracellular medium was allowed for 20 min in the absence (*Control*) or presence of verapamil (*VER*) 20 μM (n = 8), progesterone (*PROG*) 200 μM (n = 7), probenecid (*PROB*) 500 μM (n = 8), indomethacin (*IND*) 100 μM (n = 8), fumitremorgin C (*FTC*) 5 μM (n = 8) or 17ß-estradiol (*ß-EST*) 30 μM (n = 8). Shown is the [^{3}H]FA content of the cells after the efflux period, in percentage of the respective control. [^{3}H]FA uptake in control cells amounted to 0.10 ± 0.02 pmol · mg protein^{-1} (n = 8). The results are shown as arithmetic means ± SEM. *$p < 0.05$ compared to control condition

Effect of Inhibitors of ABC Transporters on Influx and Efflux of [^{3}H]FA in Primary Cultured Human Trophoblasts

Next, we examined the influence of the inhibitors of ABC transporters upon the uptake of [^{3}H]FA by human trophoblasts, both after a 6-min and after a 30-min incubation period. As can be seen in Figure 6, progesterone (200 μM) inhibited both the 6-min and the 30-min uptake of [^{3}H]FA (by 20% and 50%, respectively). The only other significant effect observed was a discrete increase in the 30-min uptake produced by 100 μM indomethacin (Fig. 5b).

Moreover, the effect of the same compounds upon [^{3}H]FA efflux was also investigated. Progesterone (200 μM), 17ß-estradiol (30 μM) and verapamil (20 μM) reduced [^{3}H]FA efflux from these cells (Fig. 7).

Discussion

The aim of this work was to investigate the putative involvement of members of the ABC superfamily of transporters on [^{3}H]FA cellular homeostasis in human trophoblasts. We focused our research on members of this family present at the placental level and which are known to accept FA and/or antifolates as substrates (the MRP1-5 transporters and BCRP) and on MDR1 or P-glycoprotein, which is also known to be present at the placental level. For this purpose, we tested the effect of known inhibitors of these transporters on both the [^{3}H]FA influx into and efflux out of BeWo cells and primary cultured human trophoblasts. The tested inhibitors were fumitremorgin C and 17ß-estradiol (inhibitors of BCRP) (Hazlehurst et al., 1999; Rabindran et al., 2000; Imai et al., 2002), probenecid and indomethacin (inhibitors of the MRP family of transporters) (Schinkel & Jonker, 2003) as well as verapamil and progesterone (inhibitors of MDR1) (Ueda et al., 1992; Barnes et al., 1996; Fromm, 2004).

The results suggest that, in BeWo cells, there is no involvement of any of the ABC transporters hypothesized (MDR1, MRP1-5 and BCRP) in [^{3}H]FA transport. Namely, P-glycoprotein (MDR1) involvement is excluded because verapamil was not able to affect either [^{3}H]FA uptake or efflux, MRP1-5 involvement was excluded because probenecid and indomethacin had no effect on either [^{3}H]FA

uptake or efflux and BCRP involvement was excluded because its selective inhibitor, fumitremorgin C, was also devoid of effect on both the influx and efflux of the vitamin. The lack of involvement of either MRP1—5 or BCRP in FA efflux in BeWo cells was unexpected as these cells are known to express these transporters (Pascolo et al., 2001; Bailey-Dell et al., 2001) and these transporters accept FA and antifolates as substrates. At the moment, we can only give two possible explanations for this observation: either these transporters are expressed but they do not contribute to [^{3}H]FA efflux or the BeWo cell cultures used in our experiments lost expression of these transporters.

Surprisingly, progesterone showed an inhibitory effect upon both [^{3}H]FA uptake and efflux in BeWo cells. Its inhibitory effect upon influx of the vitamin was concentration-dependent, the maximal inhibitory effect being about 50%. Moreover, progesterone seems to function as a competitive inhibitor of [^{3}H]FA influx because it increased the K_m of [^{3}H]FA uptake without affecting V_{max}. In relation to [^{3}H]FA efflux, progesterone showed a more discrete effect: 200 μM of this steroid reduced [^{3}H]FA efflux by only about 10%, and the effect was not concentration-dependent.

Interestingly, the results obtained using primary cultured human trophoblasts were similar to those obtained with BeWo cells. Namely, progesterone (200 μM) was also found to inhibit [^{3}H]FA uptake and efflux in human trophoblasts, inhibition of [^{3}H]FA efflux by progesterone (by 40%) being even more marked than in BeWo cells. Moreover, fumitremorgin C, probenecid and indomethacin were also devoid of effect on both [^{3}H]FA uptake and efflux.

In summary, progesterone was able to reduce both [^{3}H]FA influx and efflux in BeWo cells and primary cultured trophoblasts. Thus, the question arises: What is the mechanism involved in this inhibitory effect of progesterone? We think that inhibition of one of the ABC family members hypothesized (MDR1, MRP1-5 or BCRP) is not involved in this effect. First, progesterone caused a decrease in both the influx and efflux of [^{3}H]FA, and these transporters mediate efflux of compounds from the cells but do not mediate influx of compounds. Second, although progesterone is a well-known inhibitor of MDR1, the involvement of this transporter can be excluded for the reason mentioned above and because verapamil, another well-known MDR1 inhibitor, was devoid of effect. Thus, the mechanism involved in this inhibitory effect of progesterone remains to be elucidated.

Another family of carrier proteins that are able to transport FA is the SLC22A family of transporters (organic anion transporters) (Matherly & Goldman, 2003), and members of this family are present at the placental level (Miyazaki, Sekine & Endou, 2004). Thus, we tested the effect of *p*-aminohippuric acid, a well-known substrate/inhibitor of this family of transporters (Miyazaki et al., 2004). However, *p*-aminohippuric acid was devoid of effect on both [^{3}H]FA influx and efflux. Moreover, probenecid and indomethacin, which are also inhibitors of organic anion transporters, had no effect. Thus, we conclude that a member of the organic anion transporter family is probably not involved in [^{3}H]FA transport in BeWo cells, so inhibition of this transporter cannot explain the inhibitory effect of progesterone.

In order to further investigate the nature of the inhibitory effect of progesterone, we determined the specificity of its effect by testing the effect of some other steroids upon [^{3}H]FA uptake and efflux in BeWo cells. The inhibitory effect of progesterone upon [^{3}H]FA uptake seems to be very specific as none of the other steroids tested (ß-estradiol, testosterone, corticosterone, aldosterone, pregnenolone and estrone) had any effect. On the other hand, ß-estradiol and corticosterone also caused inhibition of [^{3}H]FA efflux, and estrone increased this parameter. These results suggest that the mechanisms involved in [^{3}H]FA uptake and efflux are not the same and/or that an additional mechanism, subject to modulation by ß-estradiol, corticosterone and estrone, is involved in the latter. Interestingly enough, [^{3}H]FA efflux from primary cultured human trophoblasts was inhibited by ß-estradiol or verapamil. Knowing that verapamil is a P-glycoprotein inhibitor and that ß-estradiol and corticosterone are organic anions and, as such, can interact with MRP1-5, the involvement of these transporters in [^{3}H]FA efflux from BeWo cells and primary cultured human trophoblasts can be hypothesized.

As stated earlier, both the RFC and folate receptors seem to be responsible for FA uptake by human placental trophoblast cells at physiological pH (Keating et al., 2005, 2006). Because folate receptors are unidirectional transporters, their involvement in the effect of progesterone was excluded. RFC, on the other hand, is a bidirectional transporter. Interestingly, the inhibitory effect of progesterone was not changed either in the presence of SITS, an inhibitor of the RFC, or in the presence of an anti-RFC antibody. These results suggest that progesterone inhibits the RFC. To our knowledge, nothing is known concerning the effect of progesterone, or steroids in general, on this transporter. The lack of effect of progesterone at an external acidic pH also argues in favor of this hypothesis as FA uptake at low pH in these cells has different characteristics from uptake at physiological pH, the presence of a low pH-operating FA transporter distinct from RFC having been recently suggested (Keating et al., 2006). Finally, the maximal inhibitory effect of progesterone upon ^{3}H-FA uptake (around 50%) is similar to the component of ^{3}H-FA uptake corresponding to the RFC in this cell line (Keating et al., 2006).

From our results, it can be hypothesized that *in vivo* FA uptake and efflux in trophoblasts is inhibited by progesterone. Although serum levels of progesterone during pregnancy (Tamimi et al., 2003) are much lower than the concentrations found to inhibit FA transport in our study, this steroid is produced mainly by the placenta, in increasing amounts up to week 40 of gestation (Strauss, Martinez & Kiriakidou, 1996; Casey & MacDonald, 1998). So, it is probable that local concentrations of progesterone at the placental level are higher than the serum levels. Interestingly enough, a negative relationship between steroid hormones and FA utilization has already been described (Worthington-Roberts, 1999). Indeed, FA deficiency sometimes develops in women taking oral steroid contraceptives (Worthington-Roberts, 1999). Moreover, it is believed that defects in the utilization of FA may be inherent to pregnancy because of the effects of high levels of steroid hormones. This happens because FA absorbed from food is converted by a series of reduction reactions to its active coenzyme form in the liver, and high sex steroid levels may interfere with this process because the liver is also the site where they are deactivated before excretion, the reactions for both the activation of FA and the deactivation of steroids involving similar biochemical mechanisms (Worthington-Roberts, 1999). In this report, we describe an inhibitory effect of progesterone on FA placental absorption, thus adding a new mechanism that can explain the negative relationship between sex steroids and FA utilization.

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