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

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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).

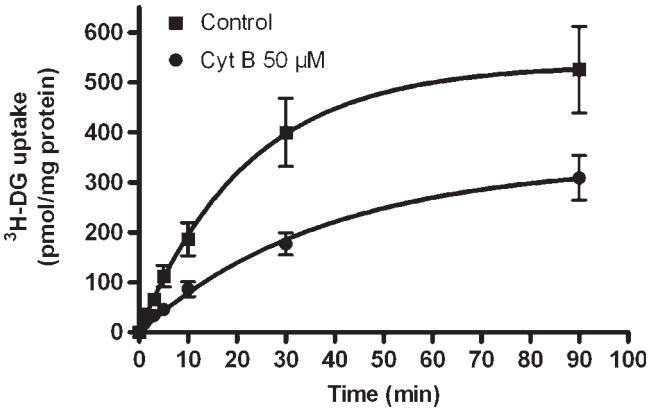

Fig. 1. **Time-course of ^{3}H-DG apical uptake by BeWo cells.** Cells were incubated at 37°C with 1 µM ^{3}H-DG for various periods of time, in the absence (control; $n = 6$) or presence of cytochalasin B 50 µM (Cyt B; $n = 5$–6). Shown are arithmetic means ± SEM.

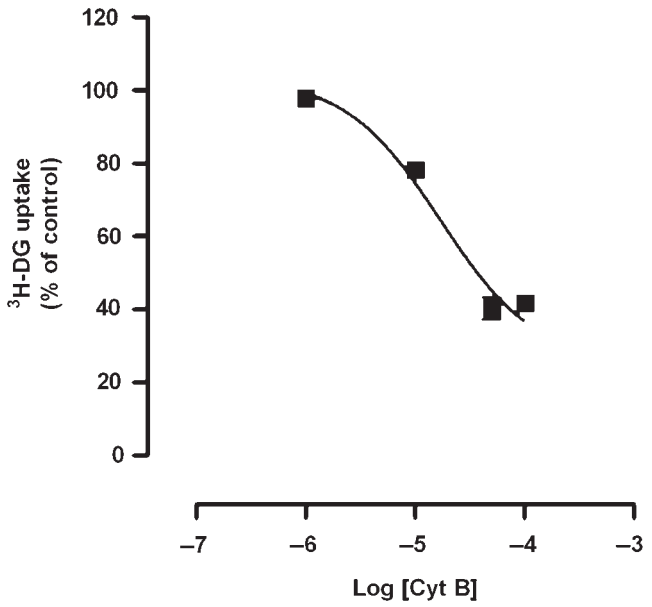

Fig. 2. **Effect of increasing concentrations of cytochalasin B upon ^{3}H-DG apical uptake by BeWo cells.** Cells were incubated at 37°C for 6 min, in the absence ($n = 14$) or presence of cytochalasin B ($n = 6$–9). Shown are arithmetic means ± SEM.

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).

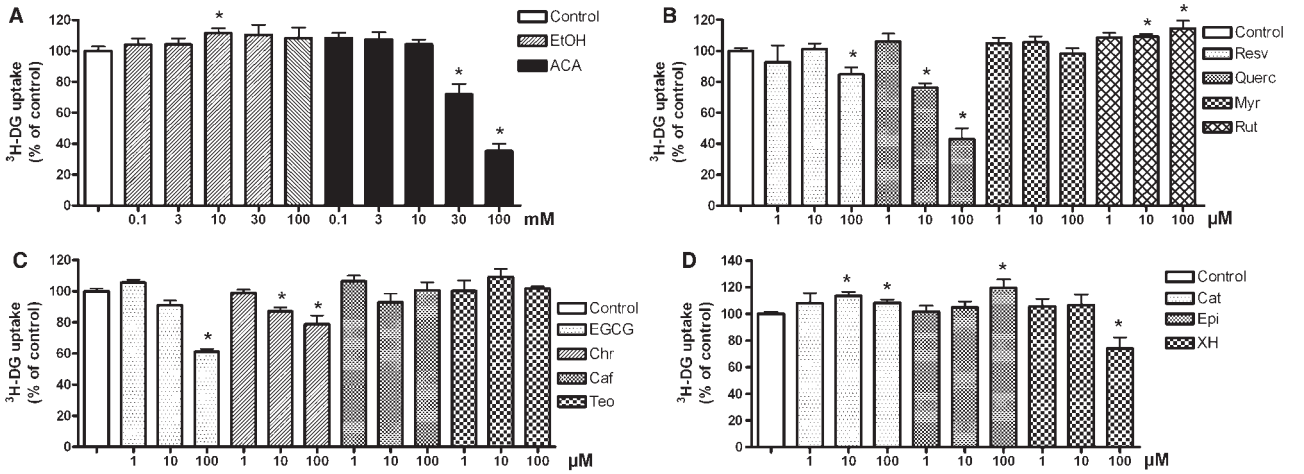

Fig. 3. **Effect of acute exposure of BeWo cells to different concentrations of (A) ethanol (EtOH; *n* = 5–6), acetaldehyde (ACA; *n* = 3–4), (B) resveratrol (Resv; *n* = 9), quercetin (Querc; *n* = 6), myricetin (Myr; *n* = 6), rutin (Rut; *n* = 6), (C) EGCG (*n* = 6), chrysin (Chr; *n* = 5), caffeine (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.

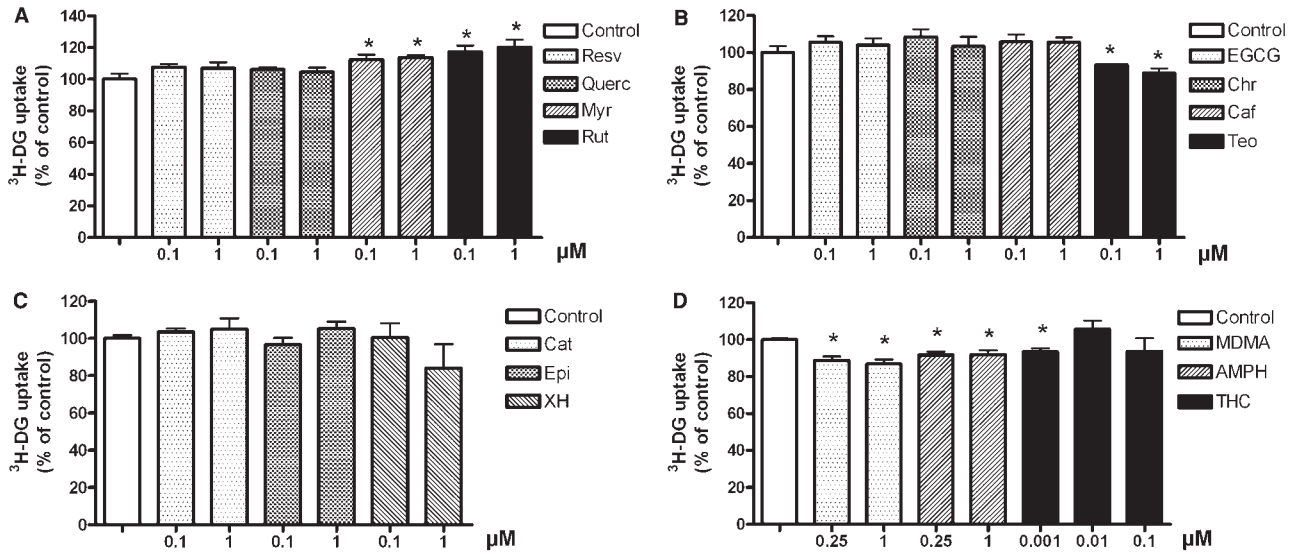

Fig. 4. **Effect of chronic exposure of BeWo cells to different concentrations of (A) resveratrol (Resv; $n = 4$), quercetin (Querc; $n = 4$), myricetin (Myr; $n = 4$), rutin (Rut; $n = 5$–6), (B) EGCG ($n = 6$), chrysin (Chr; $n = 4$), caffeine (Caf; $n = 4$), teophylline (Teo; $n = 4$), (C) catechin (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 (myricetin 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

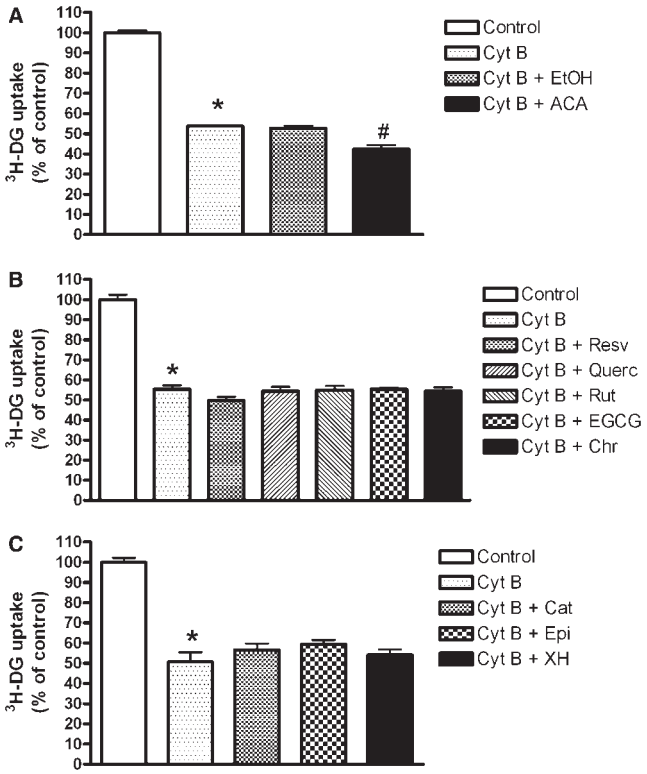

Fig. 5. **Effect of acute exposure of BeWo cells to different concentrations of (A) ethanol (EtOH; $n = 6$), acetaldehyde (ACA; $n = 6$), (B) resveratrol (Resv; $n = 9$), quercetin (Querc; $n = 9$), rutin (Rut; $n = 9$), EGCG ($n = 6$), chrysin (Chr; $n = 9$), and (C) catechin (Cat; $n = 6$), epicatechin (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.

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