Abstract

Transport characteristics of D-glucose were studied in the microvillous vesicles isolated from the human term placenta. Transport occurred by selective and rapid facilitated diffusion system which was inhibitable by phloretin and HgCl₂. The transport was dependent on a transmembrane Na⁺-gradient indicating a “secondary active transport” system operating. The transport influx was saturable and the kinetic analysis based on Hanes-Woolf plot produced a $k_t$ and $J_{max}$ value of 1.2 mM and 34 nmoles mgprotein⁻¹ min⁻¹, respectively. The efflux of D-glucose from the membrane vesicles in pre-equilibrated assay conditions showed a distinct biphasic pattern differing significantly in the half time efflux. The $t_{1/2}$ of the fast and slow components was found to be 15 sec and 660 sec, respectively. The transport showed distinct sensitivity to temperature and the Ea values both below and above the transition temperature of 37 ºC, as calculated from the Arrhenius plot were found to be 7600 and 5472 kCa⁻¹ mol⁻¹, respectively. Inhibition studies with a number of sugars for hexose transport pathway showed that the glucose epimers, phosphorylated sugars, and even the disaccharides and the pentose sugars competed effectively with D-glucose. The influx was also inhibited by a number of steroids such as progesterone, 17α-hydroxyprogesterone, testosterone and estrogen. Insulin was found to increase glucose transport in a dose-dependent fashion at a concentration of 0.2-1 unit ml⁻¹. Ouabain, dinitrophenol and nicotine strongly inhibited D-glucose uptake in the membrane vesicles.

(Nutr Hosp. 2006;21:38-46)

Key words: Glucose transport. Brush border membrane. Placenta.

CARACTERÍSTICAS DEL TRANSPORTE DE GLUCOSA A TRAVÉS DE LAS MEMBRANAS CON MICROVELLOSIDADES DE LA PLACENTA HUMANA A TERMINO

Resumen

Se estudiaron las características del transporte de la D-glucosa en las vesículas con microvellosidades aisladas de la placenta humana a término. El transporte ocurrió por un sistema de difusión selectiva y facilitada rápida que podía inhibirse por floretina y por HgCl₂. El transporte dependía de un gradiente de Na⁺ transmembrana, indicativo de un sistema operativo de “transporte activo secundario”. El flujo de entrada del transporte era saturable y el análisis cinético basado en el gráfico de Hanes-Woolf produjo una $k_t$ y una $J_{max}$ de 1,2 mM y 34 nmoles mg de proteína⁻¹ min⁻¹, respectivamente. El flujo de salida de la D-glucosa desde las vesículas de membrana en condiciones de ensayo de pre-equilibrio mostró un patrón bifásico que difería significativamente en la mitad del flujo de salida. Se halló que la $t_{1/2}$ de los componentes rápido y lento 15 seg. y 660 seg., respectivamente. El transporte mostró una sensibilidad distintiva a la temperatura, y se encontró que los valores de Ea, tanto por encima como por debajo de la temperatura de transición de 37 ºC, calculada por el gráfico de Arrhenius, fueron de 7.600 y 5.472 kCa⁻¹ mol⁻¹, respectivamente. Los estudios de inhibición con una serie de azúcares para la ruta del transporte de la hexosa mostraron que los epímeros de la glucosa, los azúcares fosforilados, e incluso los disacáridos y los azúcares pentosa, competían de forma eficaz con la D-glucosa. El flujo de entrada también se inhibió por una serie de esteroides como la progesterona, la 17α-hidroxiprogesterrona, la testosterona y los estrógenos. Se encontró que la insulina aumentaba el transporte de glucosa de manera dependiente de la dosis a una concentración de 0,2-1 unidades ml⁻¹. La ouabaina, el dinitrofenol y la nicotina inhibieron fuertemente la captación de D-glucosa por las vesículas de membrana.

(Nutr Hosp. 2006;21:38-46)

Palabras clave: Transporte de glucosa. Membrana con borde en cepillo. Placenta.
Introduction

Placenta of various species has been shown to be selectively permeable to the D-isomer of glucose and related monosaccharides in the perfused tissue, and the transport is characterized by competitive inhibition and uphill movement by counterflow. In vitro studies with the whole villous tissue have demonstrated that uptake of glucose into the syncytiotrophoblast is the initial step in the placental transfer which occurs both by bulk flow and by a mediated sodium-dependent process conformed to the requirement of Michaelis-Menten kinetics. However, the use of whole villous tissue in the study of specific characteristics of placental transport is seriously limited by the presence of various cell types and subcellular membranes, while an isolated membrane preparation would clearly facilitate such investigation. The absorptive epithelial plasma membrane, called the microvilli, from the placental syncytium can be obtained in homogeneous preparation. In further modification of the method, we have prepared closed vesicles capable of actively transporting amino acids and ions, while the purity of the membrane was established by electron microscopy, marker enzyme assays, lipid compositional analysis and SDS-PAGE profile of the membrane proteins. In this article, we report investigations on D-glucose uptake which includes substrate specificity, kinetic properties of the transport system to temperature dependence, effects of inhibitors and potential regulatory proteins. In a isolated membrane preparation would clearly facilitate such investigation. The absorptive epithelial plasma membrane, called the microvilli, from the placental syncytium can be obtained in homogeneous preparation. In further modification of the method, we have prepared closed vesicles capable of actively transporting amino acids and ions, while the purity of the membrane was established by electron microscopy, marker enzyme assays, lipid compositional analysis and SDS-PAGE profile of the membrane proteins. In this article, we report investigations on D-glucose uptake which includes substrate specificity, kinetic properties of the transport system to temperature dependence, effects of inhibitors and potential regulatory proteins. 

Materials and Methods

Collection of placenta

Placentae were collected within 30 min of vaginal delivery from mothers with uncomplicated full-term pregnancies through the courtesy of the Government General Hospital, Sector-16, Chandigarh, on written request. Strict medical ethics was followed in obtaining the placenta and the patient’s clinical history was checked from the antenatal Clinics record to avoid any pathological state such as the diabetes, hypertension and preeclampsia.

Placental microvillous (brush border) membrane preparation

The placenta was immediately placed on ice, brought to the laboratory within minutes and cut into pieces approximately 10-15 cm in diameter. Decidua and the chorionic plates were removed using a sharp microtome blade. The resulting villous tissue was washed with Earle’s balanced salt solution to remove the blood which included 23 mM CaCl₂, 1 mM MgSO₄, 5.4 mM KCl, 116 mM NaCl, 5.6 mM D-glucose, 26 mM NaHCO₃, 1 mM NaH₂PO₄ and 11 mg phenol red per liter of the solution. A 15-20 g placental tissue sample was spread out manually and then washed successively in ice-cold isotonic CaCl₂ solution, in Earle’s solution and in 40 mM Tris-HCl buffer, pH 7.4. These washings removed much of the blood from the intervillous spaces. The sample is briefly grounded with an electric food processor into small pieces of 1-5 mm in diameter and placed in an ice-cold 0.9% NaCl solution. The pH of all solutions used in the preparation was adjusted to 7.4 and except where noted otherwise, all the steps were strictly performed at 4°C. They were then gently agitated at 4°C for 30 min using a magnetic stirrer. During this procedure the chorionic villi were spread out so that their surfaces could be well irrigated. The saline is poured off and centrifuged for 10 min at 800g in a refrigerated centrifuge (g forces are average value applied to the mid-point of the tube) to remove any fragments of tissues and further centrifuged at 10,000g for 10 min to remove the larger particles and intracellular debris. This is then followed by a high speed run of 100,000 g for 60 min in a Beckman ultracentrifuge (Model L8 80) using the Ti 60 fixed angle rotor, yielded a pale yellow jelly-like pellet. The brush border membrane (pellet) was further suspended in a 2 mM Tris-Hepes buffer by homogenization in an all glass hand homogenizer and repeatedly passing through the 23 gauge needle to give a final protein concentration of approximately 2 mg.ml⁻¹. The isolated membrane was used for characterizations such as the transmission electron microscopy, marker enzyme analysis, SDS/PAGE of the membrane proteins, quantitative distribution of the lipids, as well as the studies on membrane transport.

Transport measurements

Measurement of glucose transport from the medium into the vesicles was performed under isotonic conditions. Membrane vesicles suspended in 2 mM Tris-Hepes buffer, pH 7.4 (buffer I), in the presence of absence of 130 mM NaCl were mixed with known concentration (1 mM) of 14 C-D-glucose in the same buffer with a sudden vortexing. The incubations were made at room temperature in a water bath and at indicated time intervals as short as 3 sec, the transport was stopped by diluting the medium 10-fold with ice cold phloretin solution (0.2 mM in buffer I plus 2% ethanol). The vesicles were immediately separated from the substrate medium by rapid filtration under reduced pressure on mixed ester filters (0.45 µm pore size, 0.25 mm diameter) (Whatman Limited, Maidstone, England). The membrane vesicles were harvested on the filters pre-wet with diluted buffer I and on a syringe filter holder (Whatman Limited, Maidstone, England). The vacuum for rapid filtration was genera-
Measurement of transport of the same substrate in an outward direction or egress was performed according to the method of Johnson and Smith\textsuperscript{12}. Vesicles were preloaded by equilibrating them for 1.5 h at 22°C with \(^{14}\)C-D-glucose ([S] = 1 mM) in the presence or absence of 130 mM NaCl and were immersed in an ice bath thereafter. Aliquots of 100 \(\mu\)l each were warmed to 22°C and a large aliquot (2 ml) of buffer I was rapidly added to dilute the external substrate concentration to a low level and initiate egress. Phloretin as transport blocking agent (2 ml at 0°C) was added rapidly after a desired time interval to stop the egress and the vesicles were quickly recovered as above.

Results

**Time course of D-glucose influx and egress in the placental BBM vesicles**

Fig. 1 shows that in the presence of an inwardly directed sodium gradient (initial conditions: 130 mM NaCl outside and zero inside), the influx of D-glucose in the placental BBM vesicles appeared to be extremely fast and follows a characteristic time dependent pattern. The quantity of the sugar in the vesicles reached a peak value of 32.17 nmoles.mg protein\(^{-1}\) at about 1 min which declined steadily thereafter and attained a near equilibrium value (steady state) at about 5 min. In the absence of Na\(^{+}\), the rate of uptake in the vesicles was much slower, never attained any peak accumulation and proceeded at a constant rate of about 20 nmoles.mg protein\(^{-1}\), which is only about 60% of the peak value as attained in the presence of Na\(^{+}\). The transient accumulation of the sugar in the vesicles suggests that the process is in some way linked to the inward movement of Na\(^{+}\) ions until that time when the vesicles were also equilibrated with the ion. In the subsequent experiments, the vesicles were incubated with the substrate for a period of 30 sec because it falls in the linear region of the uptake velocity.

Efflux of the substrate from the preloaded vesicles, called the 'egress' was equally fast with the internal concentration of the label falling to 75% in 20 sec and 30 sec in the presence and absence of NaCl, respectively (fig. 2). The rate then slowed abruptly as egress was studied for 10 min. The time course of egress was thus biphasic as clearly evident in the semi log plot (fig. 2 inset) that shows a halftime of 15 sec and 660 sec for the fast and slow components, respectively. Kinetic properties of the transport system were investigated with a range of substrate concentration of 1-30 mM and by measuring the initial rate of uptake of the label into the vesicles. The rate of D-glucose uptake was found to be concentration dependent over a range of 1-10 mM (fig. 3 a and b), which then saturates at higher concentrations of the monosaccharide assuming the hyperbolic kinetics typical of a protein mediated facilitated diffusion. In subsequent experiments, a substrate concentration of 30 mM was adopted ensuring a linear kinetics of the uptake process at a saturable substrate concentration. The data of uptake velocity (v) versus the substrate concentration ([S]) were transformed to a linear plot of Hanes-Woolf ([S]/v vs. [S]), and the apparent affinity constant of the uptake (k\textsubscript{m}) and the initial apparent maximum velocity (J\textsubscript{max}) were calculated from the x-intercept and the slope, respectively (fig. 3c). It follows the usual form of Michaelis-Menten equation for enzyme kinetics,

\[
\frac{[S]}{v} = \frac{[S]}{J_{\text{max}}} + \frac{k}{J_{\text{max}}}
\]

The uptake data plotted in this way produced a k\textsubscript{m} and J\textsubscript{max} value of 1.2 mM and 34.3 nmolesmg protein/min, respectively.
Inhibition by phloretin and HgC12

Fig. 4 shows that the uptake of D-glucose in BBM vesicles was found to be inhibited by phloretin (60% inhibition) and HgC12 (30%). The time course of inhibition was also followed from 15 sec to 3 min, and while the "overshoot" in D-glucose uptake was once again visible, the inhibitory effects of phloretin and HgC12 were clearly and uniformly discernible at all time points. Phloretin was therefore used as transport terminator in subsequent experiments to counter the rapidity with which the influx took place during the time lapse between incubation and filtration of membrane vesicles which was although as less as 1-2 sec.

The temperature dependence of uptake

The effect of temperature, if any, on the D-glucose uptake in the BBM vesicles was studied by following the uptake process at different temperatures ranging from 4-48 ºC (fig. 5). The results demonstrate that although the glucose uptake can be visualized even at lower temperatures, the optimum activity takes place at 37 ºC which considerably decreases thereafter at 48 ºC (25% inhibition). The uptake velocity at 0 ºC was only about 35% of that obtained at 37 ºC and at temperatures from 10-28 ºC, the uptake was found to increase linearly. The data was transformed to Arrhenius relationship of log maximum velocity to 1/T (absolute temperature).
that yielded a non-linear curve (inset). From the plot were yielded the energy of activation (E_a) values below and above the transition temperature as calculated from the slope which were found to be 7600 and 5472 kCal.mol⁻¹, respectively. The transition temperature (37 °C) was directly recorded from the plot.

Substrate specificity

The substrate specificity for D-glucose transport in the placental BBM vesicles was determined by competitive inhibition with a number of mono- and disaccharides and their structural analogues when tested in a tenfold molar excess over the substrate in the incubation medium which also contained 130 mM NaCl. Table I shows that the D-glucose transport was inhibited substantially by the glucose analogue, D-galactose, deoxyglucose, D-mannose, pentose sugars like D-arabinose, D-xylose and the disaccharides such as a-lactose, maltose and sucrose. The phosphorylated sugars, glucose-1-phosphate, glucos-6-phosphate and fructose 1,6-diphosphate also produced strong inhibition of D-glucose uptake in these vesicles. The inhibition constant (k_i) as listed in the table was calculated following the equation:

\[
\frac{v}{J_{\text{max}}} = \frac{[S]}{k_i + [S]}, \quad \text{while } k_{\text{app}} = \left(1 + \frac{[I]}{k_i}\right)
\]

The k_i values showed, as listed in table I, that the disaccharides, phosphorylated sugars and arabinose with small k_i values may prove to be the more potent inhibitors.

Effect of steroids

To study the effect of a number of steroid hormones on the uptake of D-glucose in the BBM vesicles, the membranes were preincubated for 10 min with a steroid and then the uptake studies carried out. During the trans-
port, the media contained 500 µM steroid; 30 mM substrate and 130 mM NaCl. Progesterone, 17α-hydroxyprogesterone and testosterone strongly inhibited the uptake process and 13-estradiol inhibited moderately while cholesterol produced a rather weak inhibition (table II). Also, an amount of 5% ethanol present as solvent for the steroids had no effect on glucose uptake. The inhibition constant as listed showed that the hydroxylated progesterone with 0.22 mM was the most potent inhibitor.

### Effect of insulin

Insulin was found to stimulate the glucose uptake in the placental BBM vesicles. Fig. 6 showed a dose dependent linear relationship of the increase in D-glucose uptake with an increment in insulin concentration in the incubation medium. It is also interesting to note that an extrapolation of the linear plot to the insulin concentration intersects the y-axis at a point which was identical to the Jmax value as obtained in the substrate kinetics experiment. The uptake values however, were obtained when BSA was present along with insulin, while without BSA, the value was about 29.5% less.

### Effect of inhibitors

The uptake was inhibited by a number of compounds which are known membrane perturbing agents or metabolic inhibitors (fig. 7). Ouabain, a competitive inhibitor of Na⁺/K⁺-ATPase showed about 20% inhibition of glucose uptake. 2,4-dinitrophenol (DNP), a potent metabolic inhibitor blocked the influx to as much as 50% while its para substituent remained almost without any effect. Nicotine, a well documented drug of abuse, depressed the uptake significantly up to 18% of the control value.

### Discussion

Glucose is a major fetal nutrient and understanding its transport across placenta is fundamental to the understanding of the fetal nutrition and metabolism. Since, the fetal liver is known to be deficient in gluconeogenic activities, a transplacental movement of sugar from the maternal blood remained the only source for this primary energy molecule. Transport through the...
microvillous plasma membrane of the placental syncytiotrophoblast is believed to be the first step in the transfer process. The study of D-glucose uptake in the isolated membrane vesicles in the present investigation clearly demonstrates the presence of a high affinity glucose transport which is selective and mediated by a facilitated diffusion system. The process was saturable and had a $k_t$ of 1.2 mM which is similar to that reported in a number of tissues such as the adipocyte and the cultured fibroblasts from baby hamster kidney\textsuperscript{15}. The results suggest that the monosaccharide is transiently accumulated within the vesicles by an electrogenic process dependent on the presence of an inward gradient of NaCl. The process is therefore, powered by sodium co-transport and aptly called as the “secondary active transport”\textsuperscript{16}. The transport is characterized by a huge overshoot phenomenon which is also variously reported in 10 experiments with glucose and amino acid transport in the isolated microvillous membrane vesicles of similar transport epithelia, like intestinal villous and renal proximal tubule\textsuperscript{17,18}. Outward flux of the monosaccharide is characterized by two distinct phases, a slower one preceded by an extremely rapid flux. The biphasic time course indicates the possibility of existence of two types of vesicular compartments with transport system of different affinity - one that fills and empties much faster than the other one. The transport also showed clearly its sensitivity towards temperature of incubation. The uptake was negligible at 4-15°C, proceeded maximally at 37°C and was inhibited thereafter, thus further suggesting that the process was transport protein-mediated and depended on the changes in membrane lipid microenvironment. The non-linearity in Arrhenius plot demonstrates a selective partitioning of the protein (transporter) in the ordered and liquid crystalline lipid phases. The break point as visualized directly from the plot corresponds to the membrane lipid phase transition (Tc). The Ea below the Tc (7600 kCal.mol$^{-1}$) corresponded to the transition of the native molecule to the activated state while the Ea above the Tc (5472 kCal.mol$^{-1}$) may relate to denaturation or unfolding of the higher order structures.

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Each datum represents mean ± SD of 3-4 independent observations.

The D-glucose uptake in placental BBM vesicles prefers substrates which are conformational analogues such as the D-galactose, D-mannose or the phosphorylated sugar at one or six position. It was interesting to note that a number of disaccharides also compete for the glucose binding sites. The possible explanation could be that these membranes are also richly endowed with disaccharide hydrolases\textsuperscript{19} which could provide the ready source, of the competing monosaccharide substrate. The transport system also seems to have little discrimination for the pentose sugar and the ketohexoses. The microvillous membrane glucose transport system is inhibited by a number of steroid hormones which although are synthesized \textit{in vivo} by the placenta and their local concentration cannot be told precisely, but may be presumed to be quite high. Smith and Brush\textsuperscript{20} reported a value of 1-2 µg of progesterone per g of isolated microvilli from placenta that corresponds to about $5 \times 10^{-6}$ M which is within the range of the steroids where inhibition of glucose transport was observed in the present study. Direct inhibitory effects of steroids on glucose transport had been demonstrated in other cell types such as the diethylstilbestrol in erythrocytes\textsuperscript{21}. It seems that the inhibition of glucose uptake in placental microvillous membranes from the maternal blood in ten-fold molar excess of the steroids is a part of the unique self regulatory mechanism of a tissue which is known to be autonomous. However, cholesterol, the precursor molecule for these steroids did have little effect on the uptake process. Steroid hormones have also been shown to inhibit strongly the uptake process of different amino acids in the placental membranes\textsuperscript{22}. On the other hand, the transport process was stimulated in a dose dependent fashion by insulin. Placental membranes are richly endowed with high affinity insulin receptors\textsuperscript{23-25}, although the biological effects of insulin on placenta are unknown and the membrane contains no hormone-sensitive adenylate cyclase\textsuperscript{26}. Insulin however, seemed to inhibit the amino acid movement\textsuperscript{27}. The inhibition is particularly pronounced in placental slice preparations\textsuperscript{28}. Since, insulin is rapidly degraded by placental tissues\textsuperscript{24,28} we have ad-

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Table II

\textbf{Effect of steroids on $^{14}$C-D-glucose transport in the placental BBM vesicles}

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ded BSA to the incubation medium to slow down the proteolysis of insulin.

A number of drugs, membrane perturbing agents and metabolic inhibitors have been shown to inhibit the glucose uptake in the present study. Ouabain, a cardiac glycoside which specifically inhibits the Na⁺/K⁺ ATPase by competitively binding with the K⁺-binding sites of the enzyme. The enzyme is responsible for generating the Na⁺-gradient and therefore, the inhibitory effect of the drug on glucose transport which has to be co-transported with Na⁺, is also expected. Ouabain has also been found to inhibit the amino acid transport in placental and other epithelial surfaces. Similar compounds like digoxin and ethacrynine acid which inhibited the transport ATPase, also strongly blocked the amino acid and glucose transport in placental membranes. The oxidative inhibitor, dinitrophenol inhibited the uptake while in the presence of p-nitrophenyl phosphate, the uptake of glucose appeared to maintain the near normal rate. The results are similar to that of the glucose and amino acid uptake in placental slice experiments. Dinitrophenol is also reported to inhibit the vitamin B₃ uptake in placental membranes.

The uptake is also inhibited drastically by metabolic poison, HgCl₂ and also by phloretin. Phloretin has some structural similarities to the steroids and is also known to be weakly estrogenic. It has an α-β-D-glucopyranoside in its molecule, which is used experimentally to produce glycosuria and is a potent inhibitor of glucose transport in the intestinal and renal membrane vesicles. Nicotine is a major drug of abuse, known to depress the placental amino acid transport and may be one of the factors that cause reduction in intrauterine fetal growth in smoking mothers. Nicotine considerably inhibited the glucose uptake which is surprising in view of the fact that placenta is devoid of any kind of nerve. However, it is known that placenta is rich in acetyl choline and its metabolizing enzymes. Nicotine binds to the acetyl choline/muscarnic receptor and it is believed that cholinergic regulation may form the basis of glucose and amino acid transport in placenta.

References