Riboflavin Uptake in Human Trophoblast-Derived BeWo Cell Monolayers: Cellular Translocation and Regulatory Mechanisms

SE-NE HUANG and PETER W. SWAAN
Division of Pharmaceutics, College of Pharmacy (S.-N.H., P.W.S.), and The Ohio State Biophysics Program (P.W.S.), The Ohio State University, Columbus, Ohio

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ABSTRACT

Riboflavin (vitamin B₂) is essential for fetal development and must be acquired from maternal sources. The uptake mechanism of riboflavin and the major regulatory pathways involved were characterized in a model for the placental barrier, the human choriocarcinoma cell line, BeWo. Uptake of [³H]riboflavin was saturable (K₅₀ = 1.32 ± 0.68 nM, J₅₀ = 266.63 ± 26.89 fmol/mg of protein/20 min), and was significantly reduced at low temperature and in the presence of metabolic inhibitors (azide, 2-deoxyglucose) or structural analogs. Ouabain, amoloidine, monophosphate introduced a time-dependent decrease into syncytiotrophoblasts by forskolin or 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) exerted its regulatory effect on riboflavin uptake via decreasing both J₅₀ and J₅₀ of the riboflavin uptake process (K₅₀ = 6.32 ± 1.29 nM, J₅₀ = 135.57 ± 10.42 fmol/mg of protein/20 min). In summary, we report the presence of high-affinity riboflavin transporter(s) on the microvillous membrane of BeWo cells that appears to be modulated by cellular cyclic nucleotide levels and calmodulin.

During pregnancy, the placenta not only provides a barrier separating the maternal and fetal compartments, but also serves as a transport organ supplying nutrition from the mother to the developing fetus. In humans, the maternal and fetal circulations are physically divided by a placental barrier containing the trophoblasts, villous stroma, and the fetal capillary endothelium (Rama Sastry, 1999). Transport of hydrophilic nutrients and drug molecules across the placenta is mainly controlled by the two membranes of the polarized trophoblasts, with the apical microvillous membrane in direct contact with the maternal circulation and the basal membrane facing the fetal side (van der Aa et al., 1998). To facilitate efficient passage of crucial nutrients to the fetus, trophoblasts are known to express specific nutrient transporter systems on their apical cell surfaces (Knipp et al., 1999).

Riboflavin, also known as vitamin B₂, is essential for normal cellular function. Maternal intake of riboflavin has been shown to correlate positively with fetal growth and development (Badart-Smook et al., 1997). Animal studies suggest that riboflavin deficiency during pregnancy leads to congenital abnormalities (Rivlin, 1975). Despite its critical importance to the developing fetus, the molecular mechanism and regulation of riboflavin translocation across the trophoblast are still poorly understood. Studies with perfused human placental tissues and in vivo analysis of maternal and umbilical cord plasma riboflavin levels identified a saturable uptake process on the maternal surface of the placenta putatively responsible for a 4-fold elevation in free riboflavin concentrations of fetal plasma (Dancis et al., 1985, 1988; Zempleni et al., 1992, 1995). Moe and colleagues (1994) showed that riboflavin is taken up into human syncytiotrophoblast-derived membrane vesicles by a high-affinity membrane component via a concentration-dependent, Na⁺-independent mechanism. However, the overall internalization

ABBREVIATIONS: PBS, phosphate-buffered saline; K₅₀, Michaelis-Menten-type constant; J₅₀, maximum uptake velocity; cAMP, cyclic adenosine monophosphate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; DIDS, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid; 8-Br-cAMP, 8-bromo-cyclic adenosine monophosphate; PKA, protein kinase A; IBMX, 3-isobutyl-1-methylxanthine; GMP, guanosine monophosphate; PKG, protein kinase G; pCPT-cGMP, p-chlorophenylthio-cyclic guanosine monophosphate; PKC, protein kinase C; GaM, calmodulin.
Mechanism and Regulation of Riboflavin Uptake in BeWo Cells

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The objective of this study is to disseminate the uptake mechanism of riboflavin in the human placenta and to document its intracellular regulatory pathway(s). We used a human choriocarcinoma-derived cell line, BeWo, as a model for trophoblasts. Compared with isolated membrane preparations, an intact cell line system provides a mechanistic model to integrate cellular uptake processes and intracellular events associated with modulation of transporter function. Under normal conditions, BeWo cells have been shown to exhibit morphological and biochemical features that strongly resemble proliferative cytotrophoblasts; furthermore, these cells can be induced into differentiated syncytiotrophoblasts in vitro using pharmacological agents (Wice et al., 1990; Liu et al., 1997). More importantly, several studies have shown that the characteristics of membrane transport systems expressed in BeWo cells are highly similar to those reported in normal human trophoblasts (Cool et al., 1991; Prasad et al., 1997).

Experimental Procedures

Materials. [3H]Riboflavin (20 Ci/mmol) and [14C]mannitol (60 mCi/mmol) were purchased from Sigma (St. Louis, MO) and Moravek Biochemicals (Brea, CA), respectively. Cell culture materials and buffer solutions were obtained from Life Technologies (Gaithersburg, MD). Rat tail collagen (type I) was from Becton Dickinson Labware (Bedford, MA), and the bicinchoninic acid protein assay kit was purchased from Sigma. All other chemicals were from Fisher Scientific (Pittsburgh, PA) and Sigma.

Cell Culture. BeWo cells were obtained from American Type Culture Collection (Manassas, VA). Cells with passage numbers 191 to 230 were maintained at 37°C, under 5% CO2, in complete medium consisting of F-12K medium with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were routinely maintained in tissue culture-treated 175-cm² flasks, and the culture medium was replaced every other day. The cells were harvested at 80% confluence (days 4 to 5) by exposure to a trypsin-EDTA solution (0.25% trypsin and 0.002% EDTA in Hanks’ balanced salt solution). Cell monolayers were grown on rat tail collagen-coated 12- or 24-well plates (3.8 and 2.0 cm², respectively) at a density of 5 × 10⁴ cells/cm². Confluent monolayers were formed between 3 to 5 days after seeding and were used for experiments at that time.

Uptake Experiments. Confluent BeWo cell monolayers were washed twice with warm (37°C) Dulbecco’s PBS (pH 7.4) before studies were initiated. Riboflavin uptake studies were performed at 37°C in bathing medium (Hanks’ balanced salt solution containing 25 mM glucose and 10 mM HEPES, adjusted to pH 7.4) with a final concentration of 5.0 nM [3H]riboflavin. [14C]Mannitol (0.37 μM) was incorporated in the incubation medium to determine the specificity of the washing steps. Mannitol has been shown to diffuse across the cell membrane solely by passive diffusion and, thus, can serve as a control to determine the respective effect of physical or pharmacological cell perturbations on passive and active transport processes (Huang and Swaan, 2000). After 20 min, bathing medium was aspirated, and cells were washed twice with ice-cold Dulbecco’s PBS, pH 3.9 (2 ml per well for each 2-min wash) to remove free and surface-bound riboflavin (Huang and Swaan, 2000). Finally, cells were lysed with 1% Triton X-100 solution, and the amount of dual-labeled radioactivity in cell lysates was quantitated using a Beckman liquid scintillation counter (model LS 6000IC). Cellular protein content was determined by the bicinchoninic acid method using bovine serum albumin as a standard. Modulators of signal transduction pathways were prepared in either DMSO or absolute ethanol (final concentrations of the organic solvent <1%). An identical amount of the drug-dissolving vehicle (dimethyl sulfoxide or ethanol) was incorporated in the bathing media of control experiments to determine the effect of these solvents on untreated cells. Viability of cells under all treatment regimens was monitored by the trypan blue exclusion method and was routinely between 92 and 96%.

In Vitro Differentiation Experiments. Forskolin or 8-bromo-cAMP was added to complete cell culture medium at a final concentration of 100 μM and 250 μM, respectively, followed by filter sterilization (0.22 μm). BeWo cells were grown in drug-free culture medium for 2 days to allow cell proliferation. Regular medium was replaced with drug-containing medium on the third day, and the medium was changed daily to ensure sufficient supply of nutrients. Experiments were initiated after confluence was reached at 5 days post seeding.

Data Analysis and Statistics. Kinetic uptake parameters such as the concentration at half-maximal transport velocity (Km, Michaelis-Menten-type constant), maximum uptake velocity (Jmax), and passive membrane permeability coefficient (Pm) were calculated using the NONLIN module in SYSTAT (version 8.0, SPSS, Inc., Chicago, IL) by nonlinear least-squares regression analysis of the obtained data to the general expression:

\[ J_t = \frac{J_{\text{max}} \cdot C}{K_m + C} + \frac{P_m \cdot C}{1 + \frac{C}{C_0}} \]  

(1)

where Jt is the total flux and C is the riboflavin concentration. All results are expressed as the means ± S.D. Statistical analyses between two groups were performed using Student’s t test, and one-way analysis of variance was used for single and multiple comparisons. Significant differences were reported with a confidence interval of 95%.

Results

Riboflavin Uptake Kinetics. The presence of a putative riboflavin transport system in the BeWo cell line was first determined by assessing the kinetics of riboflavin uptake. A [3H]riboflavin concentration in accordance with the free riboflavin concentration in human maternal blood (~5 nM) (Zempleni et al., 1995) was used to examine the time course of riboflavin uptake. As shown in Fig. 1, uptake increased linearly up to 20 min (r² = 0.99, rate = 10.91 fmol/min/mg of protein) and approached equilibrium at 40 min. Based on these experiments, all subsequent uptake studies and kinetic analyses were performed from data collected through 20 min.

Figure 2 shows the relationship between the uptake of [3H]riboflavin at 20 min and its concentration (1.0 nM to 1.0 μM) in the bathing solution. Analysis of total [3H]riboflavin uptake data reveals an absorption mechanism consisting of two pathways: a saturable pathway at low concentrations and an apparently nonsaturable (passive) pathway that dominates at concentrations above 250 nM (Fig. 2). [14C]Mannitol uptake ranged from 1.20 to 1.61 pmol/mg of protein at all studied [3H]riboflavin concentrations. After fitting the riboflavin data to eq. 1, an uptake system with an apparent Km of 1.32 ± 0.68 nM and a Jmax of 266.63 ± 26.89 fmol/mg of protein/20 min was identified. The dotted line represents the uptake for the saturable component calculated from the kinetic parameters. The dashed line represents the uptake for the nonsaturable flux generated from multiplying the riboflavin concentration with the passive membrane permeability coefficient (Pm, 1.19 ± 0.05 fmol/mg of protein/20 min).
Based on these data, subsequent experiments aimed to characterize the saturable riboflavin uptake component were conducted at 5.0 nM [3H]riboflavin; at this concentration, the nonsaturable transport component contributes merely 3.81 ± 0.66% to the total riboflavin uptake process (Fig. 2, inset).

**Substrate Specificity of BeWo Cell Riboflavin Uptake.** To investigate the substrate specificity of the saturable uptake process, we established the effects of various riboflavin coenzymes and structural analogs on [3H]riboflavin uptake in BeWo cells (Fig. 3). 85.6% of [3H]riboflavin uptake was blocked in the presence of 1000-fold unlabeled riboflavin.

The major coenzyme forms of riboflavin, flavin mononucleotide (FMN) and flavin adenine mononucleotide (FAD), significantly inhibited uptake of [3H]riboflavin (73.5% and 49.5%, respectively). It should be noted that FAD has significant molecular bulk attached to the 5'-hydroxy moiety on the ribose chain, namely a trihydrogenphosphosphate-adenosine group (Mw = 409) that doubles the molecular weight of this molecule compared with riboflavin. Interestingly, lumiflavin, a riboflavin analog with a methyl group substituted for the ribose side chain, was equally effective in inhibiting [3H]riboflavin uptake (48.0%); on the other hand, lumichrome, an analog that completely lacks the D-ribose side chain, showed limited affinity for the riboflavin transport system (28.4% inhibition). Addition of D-ribose to the incubation medium did not significantly affect [3H]riboflavin uptake.

The molecular structures of folates and flavins share a pterin (pyrazino[2,3-d]pyrimidine) ring system and several tricyclic antidepressants, such as chlorpromazine and amitriptyline are structurally related to riboflavin. Coadministration of these agents has been shown to enhance urinary excretion of riboflavin and accelerate tissue depletion of FAD levels in the liver (Pinto and Rivlin, 1987). However, at the cellular level, neither folate, nor chlorpromazine, nor amitriptyline had a significant effect on [3H]riboflavin uptake in BeWo cells (Fig. 3).

**Temperature and Energy Dependence.** The riboflavin uptake pathway in BeWo cells was further characterized using two additional criteria for active membrane transport, namely temperature and energy dependence. To determine temperature dependence of the transport pathway, uptake of [3H]riboflavin was performed at decreased temperatures. The rates of uptake were 3.217, 1.591, and 1.213 fmol/min at 37°, 20°, and 4°C, respectively, indicating that riboflavin uptake is temperature-dependent. At these conditions, [14C]mannitol uptake was 0.014 ± 0.002, 0.010 ± 0.001, and 0.010 ± 0.001 pmol/min at 37°, 20°, and 4°C, respectively.

Riboflavin uptake was also significantly reduced in BeWo cells pretreated with metabolic inhibitors (50 mM sodium azide/10 mM 2-deoxyglucose: 114.98 ± 11.8 fmol/mg of pro-

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**Fig. 1.** Time course of [3H]riboflavin (Rf) uptake in BeWo cell monolayers. Five-day-old BeWo cells were incubated with 5.0 nM [3H]riboflavin (circles) and 0.37 μM [14C]mannitol (triangles). Cells were washed twice with ice-cold acidic PBS, lysed with 1% Triton X-100, and measured for radioactivity at specified time points. Each value represents the mean ± S.D. of four experiments.

**Fig. 2.** Concentration dependence of [3H]riboflavin (Rf) uptake in BeWo cell monolayers. Five-day-old BeWo cells were incubated with [3H]riboflavin (1–1000 nM) and [14C]mannitol (0.37 μM). Cells were washed twice with ice-cold acidic PBS, lysed with 1% Triton X-100, and measured for radioactivity after a 20-min uptake study. Each value represents the mean ± S.D. of four experiments. Solid line represents the calculated fit of the data to eq. 1 as described under Experimental Procedures. The estimated active component (J_active) to the total riboflavin flux (J_total) is simulated with a dotted line; the estimated passive component (J_passive) is indicated with a dashed line. Inset, the enlarged portion of the figure at concentrations below 20 nM.

**Fig. 3.** Effect of riboflavin structural analogs on uptake of [3H]riboflavin (Rf). Uptake of 5 nM [3H]riboflavin and 0.37 μM [14C]mannitol were measured in the presence of 5 μM various analogs (1000-fold excess). Cells were washed twice with ice-cold acidic PBS, lysed with 1% Triton X-100, and measured for radioactivity after a 20-min uptake study. Each value represents the mean ± S.D. of four experiments. LF, lumiflavin; LC, lumichrome; Chlpmz, chlorpromazine; Amtryp, amitriptyline.
tein; control: 184.25 ± 4.25 fmol/mg of protein; 15 min pre-incubation). [14C]Mannitol uptake was not significantly affected (p > 0.05) in cells exposed to metabolic inhibitors (treated: 0.734 ± 0.10 pmol/mg of protein; control: 0.916 ± 0.08 pmol/mg of protein). These results imply that the riboflavin transport process is dependent on metabolic energy. Taken together, these results provide additional support for the presence of a carrier system that specifically mediates the uptake of riboflavin into BeWo cells.

Ion-Coupling Properties of Riboflavin Uptake. Active transport processes are generally energized by cotransport of ions (solute transport family) or adenosine triphosphate hydrolysis directly coupled to the transport system (adenosine triphosphate binding cassette [ABC]-transport family). In mammals, transport of organic solutes is primarily coupled to the electrochemical gradients of Na⁺ or H⁺ (Hediger et al., 1995). To investigate the role of sodium in riboflavin uptake in BeWo cells, we replaced Na⁺ ions in the bathing media with choline, K⁺, or Li⁺ (Table 1). None of the aforementioned substitutions led to significant inhibition of riboflavin uptake. Preincubation of BeWo cells with 1 mM ouabain, a specific Na⁺,K⁺-ATPase inhibitor, for 1 h did not affect riboflavin uptake corroborating the previous results with Na⁺-free and K⁺-enriched media. To test the potential involvement of a hydrogen-coupled transport pathway, bathing solutions with H⁺-concentrations ranging from 10⁻⁶ to 10⁻⁸ M were prepared by adjusting the pH of incubation media. Table 1 shows that uptake of riboflavin is not affected by solution pH over the hydrogen concentration range tested, suggesting that the uptake process is not driven by an inwardly directed proton gradient (Table 1). Pretreatment of cells with various concentrations of amiloride, a specific Na⁺/H⁺-exchanger inhibitor, failed to block uptake of riboflavin (Table 2), which was taken as an additional validation for both the Na⁺- and H⁺-independence of riboflavin uptake in BeWo cells.

Several studies have demonstrated that internalization of dopamine and other neurotransmitters is markedly affected by Ca²⁺ concentration (Uchida et al., 1998). To assess the calcium dependence of riboflavin uptake, studies were performed in Ca²⁺-free buffer or incubation medium with 1 mM EGTA. At the cellular level, neither treatment significantly inhibited riboflavin uptake.

Chloride is the major physiological anion with a 20-fold higher concentration in the extracellular fluid (103 mM/ℓ) relative to the intracellular environment (4 mEq/ℓ) (Guyton and Hall, 2000). Studies have shown that active transport of neurotransmitters, including serotonin, γ-aminobutyric acid and some β-amino acids, requires specific coupling of a downhill Cl⁻ gradient into the cells (Griffith and Sansom, 1998). To test the hypothesis that riboflavin uptake depends on selective cotransport of Cl⁻, experiments were conducted in bathing media containing salts of alternative organic and inorganic monovalent anions (gluconate, iodide, and isocyanate). Substitution of Cl⁻ ions with I⁻, SCN⁻, or gluconate significantly reduced the uptake of riboflavin in BeWo cells (21.5, 33.2, and 15.6% respectively; Table 1). Analysis of concomitant [14C]mannitol uptake reveals that substitution of Cl⁻ ions does not significantly affect the passive diffusion of this marker molecule, indicating the specificity of this treatment regimen on the riboflavin transport system. Effects of anion exchange inhibitors and general organic anion transporter inhibitors on riboflavin uptake in BeWo cells were also investigated (Table 2). Pretreatment of BeWo cells with various concentrations of probenecid (a general organic anion inhibitor) or furosemide (a Na⁺,K⁺-2Cl⁻ cotransporter inhibitor) did not decrease riboflavin uptake, whereas 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), a membrane impermeable anion-exchanger inhibitor, repeatedly reduced riboflavin uptake to 20% to 30%. Control [14C]mannitol data indicate DIDS inhibition to be specific for the riboflavin uptake system.

Riboflavin Uptake in Syncytiotrophoblasts. Syncytiotrophoblasts, the differentiated phenotype of cytotrophoblasts, are the main functional units of the term placenta (Knipp et al., 1999). More importantly, several studies have demonstrated that induction of cell differentiation was fre-

### Table 1

<table>
<thead>
<tr>
<th>Influence of ions* and pH† on riboflavin uptake in BeWo cell monolayers</th>
<th>[3H]Rf Uptake (% of Control)</th>
<th>[14C]Ma Uptake (% of Control)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100.00 ± 5.15</td>
<td>100.00 ± 8.48</td>
</tr>
<tr>
<td>Choline → Na⁺</td>
<td>91.33 ± 4.33</td>
<td>99.66 ± 14.22</td>
</tr>
<tr>
<td>K⁺ → Na⁺</td>
<td>101.73 ± 3.89</td>
<td>111.24 ± 21.78</td>
</tr>
<tr>
<td>Li⁺ → Na⁺</td>
<td>101.97 ± 5.79</td>
<td>105.65 ± 23.62</td>
</tr>
<tr>
<td>1 mM Ouabain</td>
<td>93.08 ± 8.61</td>
<td>101.74 ± 13.70</td>
</tr>
<tr>
<td>Ca²⁺-free buffer</td>
<td>100.09 ± 2.45</td>
<td>109.89 ± 8.93</td>
</tr>
<tr>
<td>1 mM EGTA</td>
<td>95.67 ± 5.12</td>
<td>91.63 ± 10.06</td>
</tr>
<tr>
<td>Control</td>
<td>100.00 ± 8.73</td>
<td>100.00 ± 18.66</td>
</tr>
<tr>
<td>I⁻ → Cl⁻</td>
<td>78.49 ± 6.18***</td>
<td>91.18 ± 8.21</td>
</tr>
<tr>
<td>SCN⁻ → Cl⁻</td>
<td>66.85 ± 5.03***</td>
<td>93.32 ± 13.16</td>
</tr>
<tr>
<td>Gluconate⁻ → Cl⁻</td>
<td>84.39 ± 5.23**</td>
<td>80.54 ± 9.32</td>
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### Table 2

<table>
<thead>
<tr>
<th>Effect of anion-exchange inhibitors and organic anion transport inhibitors on riboflavin uptake in BeWo cell monolayers*</th>
<th>[3H]Rf Uptake (% of pH 7)</th>
<th>[14C]Ma Uptake (% of pH 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3</td>
<td>93.94 ± 1.40</td>
<td>95.06 ± 11.35</td>
</tr>
<tr>
<td>pH 4</td>
<td>96.95 ± 4.00</td>
<td>88.24 ± 10.82</td>
</tr>
<tr>
<td>pH 5</td>
<td>97.12 ± 3.72</td>
<td>92.49 ± 5.13</td>
</tr>
<tr>
<td>pH 6</td>
<td>94.26 ± 4.59</td>
<td>98.98 ± 12.72</td>
</tr>
<tr>
<td>pH 7</td>
<td>100.00 ± 3.51</td>
<td>100.00 ± 11.85</td>
</tr>
<tr>
<td>pH 8</td>
<td>96.86 ± 4.93</td>
<td>94.90 ± 5.75</td>
</tr>
</tbody>
</table>

** p < 0.01, *** p < 0.001 versus control.
* Five-day-old BeWo cells were incubated with 5 mM [3H]riboflavin (RF) and 0.37 μM [14C]mannitol (Ma) for 20 min either in control bathing medium (137 mM NaCl, 5.4 mM KC1, 5.4 mM CaCl₂, 1 mM MgSO₄, 25 mM glucose, 10 mM HEPES) or in buffers in which NaCl was replaced with 137 mM of various inorganic salts. Ca²⁺-free buffer contains the same composition of salts as in the control buffer, except 0.1 mM EDTA and no CaCl₂ were incorporated. After a 20-min uptake study, cells were washed twice with ice-cold acidic PBS, lysed with 1% Triton X-100, and measured for radioactivity. Each value represents the mean ± S.D. of four experiments.

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* p < 0.05 versus untreated control.
5 mM [3H]riboflavin (RF) and 0.37 μM [14C]mannitol (Ma) were added to 5-day-old confluent BeWo cells pretreated with these inhibitors (10 min, 37°C). Cells were washed twice with ice-cold acidic PBS, lysed with 1% Triton X-100, and measured for radioactivity after a 20-min uptake study. Each value represents the mean ± S.D. of four experiments.

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quently accompanied with changes in membrane transporter activities (Furesz et al., 1993; Ogura et al., 2000). The majority of BeWo cells in culture express the cytotrophoblasts phenotype; however, they can undergo in vitro differentiation into syncytiotrophoblasts upon stimulation with cAMP modulators (Wice et al., 1990; Liu et al., 1997).

To examine the uptake activities of riboflavin in the syncytiotrophoblast, two cAMP modulators with different mechanisms of action were used to induce differentiation of BeWo cells. Forskolin activates adenyl cyclase, and treatment with this drug has been shown to increase linoleic acid transport in BeWo cells (Liu et al., 1997). 8-Bromo-cyclic AMP (Br-cAMP), a membrane permeable cAMP analog, has been demonstrated to stimulate the expression of the placental facilitative glucose transporter-1 (Ogura et al., 2000). Incubation of BeWo cells with 100 μM forskolin or 250 μM Br-cAMP for 24 h resulted in a significant decrease in [3H]riboflavin uptake (Fig. 4), although noticeable features of morphological differentiation were not found under light microscopy (results not shown). Continuous drug exposure induced a time-dependent reduction in [3H]riboflavin uptake. After 72 h treatment, both agents induced 27% reduction in riboflavin uptake that was accompanied with dramatic morphological changes. Consistent with previous reports (Wice et al., 1990; Liu et al., 1997), differentiated BeWo cells exhibit an increased number of enlarged cells with fused nuclei and large intercellular openings closely resembling syncytiotrophoblasts (data not shown). [14C]Mannitol uptake in syncytiotrophoblast cells was not significantly different from untreated BeWo cells, suggesting that chemically induced differentiation specifically affects the riboflavin transport system (data not shown).

Parallel sets of cells were further used in these studies to assess whether the specificity of riboflavin uptake was modified during in vitro differentiation. Competitive studies were conducted by measurement of [3H]riboflavin uptake in the presence and absence of 1000-fold of unlabeled riboflavin. Compared with the control cytotrophoblasts, no significant difference was found in the differentiated BeWo cells during various time periods (control: 26.83 ± 4.72% and drug-treated cells: 26.92 ± 5.47%, p = 0.974).

**Fig. 4.** Time-dependent effect of forskolin and 8-Br-cAMP on [3H]riboflavin uptake (Rf) in BeWo cell monolayers. Two-day-old BeWo cells were grown in the presence of 100 μM forskolin or 250 μM 8-Br-cAMP for the time indicated. 5 nM [3H]riboflavin and 0.37 μM [14C]mannitol were added to cells for a 20-min uptake study. Each value represents the mean ± S.D. of four experiments. **p < 0.01, ***p < 0.001 versus untreated controls.

Effect of Cyclic Nucleotide-Dependent Pathways on Riboflavin Uptake. The reduction of riboflavin uptake by cAMP modulators suggests a role of cAMP-dependent protein kinase A (PKA) pathways in the regulation of membrane riboflavin transport. To directly test this hypothesis, riboflavin uptake studies were conducted in the presence of 3-isobutyl-1-methyl-xanthine (IBMX), a PKA pathway activator that prevents degradation of cAMP by inhibiting cyclic nucleotide phosphodiesterase (Shafer et al., 1998). Short-term IBMX incubation (1 h) resulted in immediate and significant inhibition of [3H]riboflavin uptake in BeWo cell monolayers (Fig. 5A). To further investigate the mechanism involved in the IBMX-mediated reduction, the riboflavin transporter activity in control and IBMX-treated BeWo cells was analyzed (Fig. 5B). Kinetic analysis revealed that IBMX-induced inhibition of riboflavin uptake is accompanied by changes in Kt, as well as in Jmax. IBMX treatment increased the Kt from 1.10 ± 0.05 nM to 6.32 ± 1.29 nM. Furthermore, the Jmax was markedly reduced from 281.80 ± 2.99 fmol/mg of protein/20 min to 135.57 ± 10.42 fmol/mg of protein/20 min in IBMX-treated cells.

Since IBMX exhibits broad-spectrum activity toward several subtypes of cyclic nucleotide phosphodiesterases (Schudt et al., 1996), IBMX-mediated reduction of riboflavin uptake may originate from its effect on modulation of the cGMP-dependent protein kinase G (PKG) pathways. To examine the involvement of the PKG pathway, we conducted uptake studies in the presence of pCPT-cGMp, a membrane permeable analog of cGMP. Pretreatment with pCPT-cGMP caused significant attenuation of riboflavin uptake in BeWo cell monolayers (Table 3). Coincubation of pCPT-cGMP and IBMX potentiated the uptake reduction to 43.7% (p < 0.001) and 15.3% (p < 0.05), compared with that of cells treated with pCPT-cGMP or IBMX alone, respectively. Analysis of concomitant [14C]mannitol uptake reveals that treatment of BeWo cells with the aforementioned pharmacological agents does not significantly affect the passive diffusion of this marker molecule, implying specificity of this treatment regimen on the riboflavin transport system.

**Effect of Cyclic Nucleotide-Dependent Pathways on Riboflavin Uptake.** The reduction of riboflavin uptake by cAMP modulators suggests a role of cAMP-dependent protein kinase A (PKA) pathways in the regulation of membrane riboflavin transport. To directly test this hypothesis, riboflavin uptake studies were conducted in the presence of 3-isobutyl-1-methyl-xanthine (IBMX), a PKA pathway activator that prevents degradation of cAMP by inhibiting cyclic nucleotide phosphodiesterase (Shafer et al., 1998). Short-term IBMX incubation (1 h) resulted in immediate and significant inhibition of [3H]riboflavin uptake in BeWo cell monolayers (Fig. 5A). To further investigate the mechanism involved in the IBMX-mediated reduction, the riboflavin transporter activity in control and IBMX-treated BeWo cells was analyzed (Fig. 5B). Kinetic analysis revealed that IBMX-induced inhibition of riboflavin uptake is accompanied by changes in Kt, as well as in Jmax. IBMX treatment increased the Kt from 1.10 ± 0.05 nM to 6.32 ± 1.29 nM. Furthermore, the Jmax was markedly reduced from 281.80 ± 2.99 fmol/mg of protein/20 min to 135.57 ± 10.42 fmol/mg of protein/20 min in IBMX-treated cells.

Involvement of Protein Kinase C (PKC)- and Calmodulin-Mediated Pathways. The apparent PKA- and PKG-dependent uptake of riboflavin suggested a potential involvement of multiple signal transduction pathways in the regulation of membrane riboflavin transporter activity. Therefore, we further assessed the influence of PKC- and calmodulin (CaM)-mediated pathways on riboflavin uptake in BeWo cell monolayers. No significant change in riboflavin uptake was found upon treatment of BeWo cells with phorbol-12-myristate-13-acetate, a well known protein kinase C activator or chelerythrine, a selective protein kinase C inhibitor (Table 4).

**Involvement of Protein Kinase C (PKC)- and Calmodulin-Mediated Pathways.** The apparent PKA- and PKG-dependent uptake of riboflavin suggested a potential involvement of multiple signal transduction pathways in the regulation of membrane riboflavin transporter activity. Therefore, we further assessed the influence of PKC- and calmodulin (CaM)-mediated pathways on riboflavin uptake in BeWo cell monolayers. Mixed effects were observed in BeWo cells exposed to modulators of CaM-mediated pathways. Pretreatment of cells with calmidazolium and W-13, two specific but structurally different CaM antagonists, resulted in marked concentration-dependent reduction on riboflavin uptake (Table 4). In contrast, neither KN-93, a selective Ca2+/CaM-dependent protein kinase II inhibitor, nor KN-92, a negative control of KN-93, exhibited significant effect. Further kinetic analysis of riboflavin uptake in the presence and absence of calmidazolium was performed to elucidate
the riboflavin affinity to its transporters changed from 0.03 fmol/mg of protein/20 min; calmidazolium-treated maximal uptake velocity dropped 59.8% (control: 295.4 ± 6.6).

BeWo cells were incubated with varying concentrations of IBMX for 1 h, experiments. The uptake of [3H]riboflavin in IBMX-treated cells. [3H]Riboflavin (1–20 nM) and [14C]mannitol (0.37 nM) were added to confluent BeWo cells pretreated with these inhibitors (1 h, 37°C). Cells were washed twice with ice-cold acidic PBS, lysed with 1% Triton X-100, and measured for radioactivity after a 20-min uptake study. Each value represents the mean ± S.D. of four experiments.

**Fig. 5.** Effect of IBMX on [3H]riboflavin (Rf) in BeWo cell monolayers. A, BeWo cells were incubated with varying concentrations of IBMX for 1 h, after which a 20-min uptake experiment was performed with 5 nM [3H]riboflavin and 0.37 μM [14C]mannitol. B, concentration-dependent uptake of [3H]riboflavin in IBMX-treated cells. [3H]Riboflavin (1–20 nM) and [14C]mannitol (0.37 nM) were added to 5-day-old control cells (circles) or cells pretreated with 5 mM IBMX for 1 h (triangles). The passive diffusion component was obtained using eq. 1 under Experimental Procedures and subtracted from total uptake values to present only the saturable uptake process. Each value represents the mean ± S.D. of four experiments.

The placental membrane mediates the entry of maternal nutrients into the fetal circulation. Although transplacental transport of riboflavin has been extensively studied in the perfused placental tissues, little is known regarding its cellular translocation and regulation mechanism across the cytotrophoblasts. The current study reports the existence of high-affinity riboflavin transporter(s) on the microvillous membrane of the BeWo human placental choriocarcinoma cell line. Supporting evidence for this active transport mechanism include: 1) a significant dependence of riboflavin uptake with temperature, 2) reduction of uptake in the presence of metabolic inhibitors, 3) a saturable uptake component, and 4) inhibition of riboflavin uptake in the presence of structural analogs. Riboflavin has relatively high affinity for its uptake pathway (Kₐ = 1.32 nM), suggesting the presence of a receptor-based translocation mechanism, which may resemble the receptor-mediated riboflavin pathway in Caco-2 cells we recently documented (Huang and Swaan, 2000).

The essential structural requirements for specific interaction between riboflavin and its placental transporters were deduced from inhibition studies with structural analogs. The isoalloxazine ring appears to have an essential role in ligand-transporter interactions, as evidenced by the finding that D-ribose does not affect riboflavin uptake (Fig. 3). This interpretation is further substantiated by significant uptake inhibition of lumiflavin and lumichrome, two riboflavin analogs that lack a D-ribose chain. Moreover, the lack of affinity of tricyclic antidepressants and folate demonstrates that the transporter uniquely recognizes flavin isoalloxazine moieties. Compared with unlabeled riboflavin, analogs with ex-
to the Na

monolayers. Furthermore, riboflavin uptake is not sensitive to DIDS-sensitive anion exchanger, a DIDS-sensitive Cl

membrane have been identified, namely an electroneutral H

and other tissues (Said and Ma, 1994; Kumar et al., 1998; Said et al., 1998), no obvious requirements for Na

, Ca

, or H

were found on the uptake of riboflavin in BeWo cell monolayers. Furthermore, riboflavin uptake is not sensitive to the Na

,K

,ATPase inhibitor (ouabain) and the Na

,H

exchanger inhibitor (amiloride), confirming the sodium and proton gradient-independent behavior of this transport system.

Interestingly, substitution of chloride ions in bathing media with other monovalent anions resulted in an incomplete, yet significant, reduction of riboflavin uptake. Comparable levels of riboflavin uptake attenuation by DIDS, a well-documented anion exchanger/chloride channel inhibitor, further corroborates the potential role of Cl

ions in the epithelial uptake of riboflavin. Unlike in other epithelia, Cl

transfer across the placental membrane is Na

independent (Illsley et al., 1988). Consequently, this explains our observation that furosemide, a Na

,K

-2 Cl

cotransporter inhibitor, has no effect on riboflavin uptake in BeWo cells. Three dominant mechanisms of Cl

transfer across the placental microvillus membrane have been identified, namely an electroneutral DIDS-sensitive anion exchanger, a DIDS-sensitive Cl

conductance, and a DIDS-insensitive voltage-dependent Cl

conductance (Stulc, 1997). The Cl

dependence of riboflavin uptake in our studies is not attributed to the placental DIDS-sensitive anion exchanger. This is evidenced by the: 1) capability of the placental anion exchanger to transfer other monovalent anions, including I

and SCN

(Stulc, 1997); and 2) insignificant inhibition of riboflavin uptake by probenecid. Taken together, our results suggest for the first time that placental riboflavin uptake may be linked to a DIDS-sensitive Cl

conductance. It should be noted that riboflavin uptake is not solely coupled to an electrochemical Cl

gradient, since removal of Cl

does not result in complete loss of uptake activity. The underlying mechanism behind Cl

-coupled riboflavin uptake requires further investigation.

It has been shown that differentiation of cytotrophoblasts is accompanied by changes in membrane transporter activities (Furesz et al., 1993; Liu et al., 1997). Our data show that riboflavin uptake in differentiated syncytiotrophoblasts induced by forskolin or 8-bromo-cAMP is significantly lower relative to proliferating cytotrophoblasts. Previously, Furesz and colleagues (1993) have reported a decreased alanine uptake via sodium-dependent neutral amino acid transporters (ASC system) in differentiated BeWo cells (Furesz et al., 1993). The reduced activities were attributed to apparent loss of the transporter proteins during extensive membrane remodeling processes throughout syncytiotrophoblast maturation and polarization. Currently, we cannot rule out the possibility that diminished riboflavin uptake is a consequence of drug-induced modification of other cellular events. The specificity of riboflavin transport in both undifferentiated and differentiated cell types remains unaltered; thus, reduced uptake could be attributed to decreased expression of the transport system during differentiation as a result of diminishing metabolic demands. Alternatively, higher riboflavin requirements in cytotrophoblast stem cells may simply reflect a greater demand of nutrients for cellular proliferation.

Several studies have demonstrated that the activity of membrane transport systems are rapidly regulated by the major signaling pathways, namely protein kinase A-, C-, and Ca

/calmodulin-mediated pathways (Racke et al., 1998; Braiman et al., 1999). Our results show that riboflavin uptake in BeWo cells appears to be modulated by cellular levels of cyclic nucleotides and CaM, but not by the protein kinase C pathway. Pretreatment of cells with PKA pathway stimulants or CaM antagonists caused marked decreases in uptake velocity ($J_{max}$) and affinity ($K_i$) of riboflavin absorption. The precise nature of the target sites in these signaling pathways is unknown, since the molecular identity of the riboflavin transport system(s) are yet to be defined. The observation that short-term incubation (1 h) resulted in pronounced effects on riboflavin uptake eliminate the possible involvement of de novo biosynthesis of transporter mRNAs or proteins. Protein kinase A phosphorylation has been shown to reduce the Cl

conductance across placental microvillus membrane (Placchi et al., 1991), which allows us to speculate that PKA modulators exert their effect by changing the Cl

coupling properties of riboflavin uptake.

Most signal transduction pathways are involved in diverse and critical functions of cells (Alberts et al., 1994). Using two CaM antagonists of different structural classifications enabled us to identify the specific involvement of CaM. Ineffectiveness of KN-93, a selective CaM-kinase II inhibitor, on blockage of riboflavin uptake further excludes the role of this ubiquitous CaM-dependent kinase in the CaM-mediated signaling processes. The finding that activators targeting three distinct components of the PKA cascade all lead to reduction of riboflavin uptake strongly suggests a significant relationship between intracellular levels of cAMP and cellular trans-
location of riboflavin. Interestingly, elevation of cGMP via pCPT-cGMP also results in a decrease of riboflavin uptake. Synergistic inhibition from coinubcation of pCPT-cGMP and IBMX further supports the involvement of both cyclic nucleotide secondary messengers in riboflavin uptake in BeWo cells. In contrast to cAMP, relatively little is known about the mechanism of action of cGMP on function of membrane transporters (Foreman and Johansen, 1996). Further studies are underway to disseminate the precise role of the PKG pathway and its involvement in the regulation in riboflavin uptake.

In a series of studies, Said and coworkers (1994) found that riboflavin uptake in various tissues is modulated by different regulatory mechanisms. In Caco-2 cells, activation of the PKA pathway leads to down-regulation of riboflavin uptake (Said et al., 1994), whereas inhibition of the CaM-mediated processes attenuates uptake activities in renal, hepatic cell lines (Kumar et al., 1998; Said et al., 1998). Currently, the physiological significance behind the multiple-signalng regulatory mechanism involved in placental riboflavin uptake is not understood. Extensive cross-talk between cAMP and calmodulin- mediated signal transduction pathways exists at several levels of cellular control mechanism (Alberts et al., 1994); therefore, the observed cyclic nucleotide- and CaM-mediated reduction of riboflavin uptake in BeWo cells might be a manifestation of intertwined regulation of these processes. A similar phenomenon has been observed in the modulation of serotonin and dopamine transporter activities (Cook et al., 1991; Jayanthi et al., 1994; Ramamoorthy et al., 1995; Zhu et al., 1997; Batchelor and Schenk, 1998). In both cases, activation of PKA pathway increases the mRNA transcripts of transporters, but stimulation of PKC pathway impairs transporter activities without influencing the mRNA levels and membrane densities of transporters.

In summary, our experiments demonstrate the presence of a high-affinity riboflavin transporter system exists in the BeWo cell line. This active transport system does not require coupling to an electrochemical Na+ gradient, corroborating previous observations in microvillous membrane vesicles isolated from human syncytiotrophoblasts (Moe et al., 1994). We report, for the first time, that riboflavin uptake in BeWo cells is partly associated with a DIDS-sensitive Cl- channel from human syncytiotrophoblasts (Moe et al., 1994). We previous observations in microvillous membrane vesicles isolated from human placental choriocarcinoma cell line (JAR). J Biol Chem 266:15750–15757.


Address correspondence to: Dr. Peter W. Swaan, Division of Pharmaceutical Sciences, College of Pharmacy, 500 W. 12th Ave., Columbus OH 43210-1292. E- mail: swaan.1@osu.edu