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Human intestinal cell line Caco-2: a useful model for studying cellular and molecular regulation of biotin uptake

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The mechanisms of enterocyte and molecular regulation of biotin uptake are poorly understood. An intestinal cell line possessing the transport characteristics of native intestinal cells is highly desirable to investigate the finer details of the cellular processing and molecular regulation of biotin transport. In the present study, we investigated the uptake of the water-soluble vitamin biotin by a human intestinal cell line Caco-2. Uptake of both low (4 nM) and high (20 μM) concentrations of biotin by confluent monolayers of Caco-2 cells was appreciable and linear for up to 10 min of incubation. Replacement of Na^+ in the incubation medium with other monovalent cations – K^+, choline, Li^+ and NH_4^+ – caused a significant inhibition of biotin uptake; a relatively lesser inhibition was seen with Li^+. Initial rate of uptake of biotin was temperature-dependent and saturable as a function of concentration at 37°C but not at 4°C. The V_{max} and apparent K_m of the temperature-dependent saturable process were 520 pmol/mg protein per min and 9.5 μM, respectively. The addition of unlabeled biotin and the structural analogue desthiobiotin to the incubation media caused a significant inhibition of the uptake of [^{3}H]biotin. The inhibitory effect of desthiobiotin was competitive in nature with an inhibition constant (K_i) of 41 μM. Biocytin, on the other hand, was a weak inhibitor and biotin methyl ester and diaminobiotin did not have any effect. Pretreatment of Caco-2 cells with the monovalent cation ionophore gramicidin and the Na^+,K^+-ATPase inhibitor ouabain caused significant inhibition of biotin uptake. Pretreatment with the K^+ ionophore valinomycin did not affect biotin uptake. Using the 'Activation Method', the stoichiometric ratio of biotin$^-$ to Na^+ coupling was found to be 1 : 1. Growing confluent Caco-2 cells in a biotin-deficient environment resulted in rapid up-regulation of biotin transport with a marked increase (258%) in the V_{max} of biotin uptake. These findings demonstrate that biotin uptake by Caco-2 cells is via a carrier-mediated system. This system is temperature-dependent, driven by Na^+-gradient and is regulated by the substrate level. These in-vitro findings are very similar to and further confirm previous findings in human and animal studies and dispute other findings previously reported for Caco-2 cells; the present study also demonstrates the suitability of this system for further characterization of the cellular and molecular regulation of biotin uptake.

Introduction

Biotin, a water-soluble micronutrient, is essential for normal cellular functions, growth and development. The vitamin is involved in a variety of critical metabolic reactions including biosynthesis and catabolism of fatty acids, gluconeogenesis and catabolism of amino acids. Mammals must obtain biotin via intestinal absorption because they cannot synthesize the vitamin endogenously. Our current knowledge of the intestinal transport mechanisms of biotin was derived from studies using intact intestinal tissue preparations or isolated membrane vesicles derived from native intestinal tissue [1–6]. While these studies have demonstrated an existence of a sodium-dependent apical membrane carrier for intestinal uptake of biotin, the enterocyte and molecular regulation of biotin uptake is poorly understood, in part, due to a lack of good cellular model of biotin transport. A cell culture system of high viability, homogeneity and polarization for studying biotin transport has not been described. Identifying such an in vitro system (particularly one of human origin) will provide a powerful tool for studying the cellular and molecular regulatory events that govern the transport phenomenon of biotin in a homogeneous cell population, and will circumvent the problems associated with obtaining sufficient (and suitable) human intestinal tissue and performing certain studies in humans. Such a system will also allow studying the effects of prolonged exposure to agents or particular set of conditions on the enterocyte uptake process of biotin isolated from other systemic factors.

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Caco-2 cells, a human-derived colonic carcinoma cell line, have unique properties in that when grown as confluent monolayers they differentiate spontaneously in culture and exhibit many structural and functional properties of mature small intestinal absorptive cells including a well defined apical brush border membrane and junctional complexes [7–9]. Because of that, Caco-2 cells have been used extensively in recent years to characterize the mechanisms and regulation of uptake processes of variety of substrates as well as to study other intestinal functions [10–13]. A recent study by Cogburn et al. [14] demonstrated an absence of apical membrane biotin carrier in the Caco-2 cells. They showed that Caco-2 uptake of biotin was mediated through a passive process.

The major goal of the present study was to investigate the characteristics of biotin uptake by confluent monolayers of Caco-2 cells across the apical membrane. In particular, we were interested in determining whether biotin uptake was mediated by apical membrane transporter as demonstrated for native intestinal absorptive cells [2–6] or through a passive process as shown by Cogburn et al. [14]. Our results indicate the existence of an apical membrane uptake carrier system for biotin in this cell line, and we describe various factors and mechanisms which affect biotin uptake by the Caco-2 cells.

Materials and Methods

[8,9(n)-^{3}H]Biotin (spec. act. 40–50 Ci/mmol; radiochemical purity of $> 97\%$) and [^{14}C]inulin (spec. act. 1–3 mCi/g) were purchased from NEN, Boston, MA. Dulbecco's modified Eagle medium, trypsin, fetal bovine serum (FBS) and other cell culture ingredients were purchased from Irvine Scientific (Santa Ana, CA). All other chemicals and reagents were of analytical quality and were purchased from commercial sources.

The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (passage 17) (Rockville, MD) and were used for uptake studies between the 19th and 24th passages. Cells were routinely grown in 75-cm^2 plastic flasks (Corning) in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, supplemented with 1% non-essential amino acids, 10% FBS, 100 U/ml penicillin, 50 U/ml streptomycin and 0.25 μg/ml amphotericin B. Cells were maintained at 37°C in a 5% CO_2 atmosphere. The culture media were changed every 2 days. The 70–90% confluent cells were subcultured by trypsinization with 0.25% trypsin and 0.9 nmol/l ethylenediamine tetraacetic acid in Ca^{2+}-free and Mg^{2+}-free phosphate buffered saline solution. For the uptake experiments, Caco-2 cells were plated onto 12-well plates at a concentration of $5 \cdot 10^5$ cells per well. All uptake studies were carried out between 7 to 10 days following confluence. Cultures were examined on a regular basis under an inverted light microscope to monitor growth and contamination. Cell viability was also monitored regularly (Trypan blue dye exclusion) and ranged between 92 and 96%.

For biotin deficiency studies, biotin deficient condition was created by adding avidin (2 mg/100 ml) to the growth media 48 h prior to biotin uptake studies. This amount of avidin represents an excess of what is needed to bind the total amount of biotin in the growth media (estimated at 4.2 μg/100 ml). There was no significant difference in appearance or viability between the cells grown in the biotin deficient or sufficient (normal) media.

Uptake studies were performed at 37°C (unless otherwise stated). The incubation solution consisted of 1 ml pre-warmed Krebs-Ringer phosphate buffer (in mM: NaCl 123, KCl 4.93, $MgSO_4$ 1.23, $CaCl_2$ 0.85, glucose 5, glutamine 5, NaH_2PO_4 20 (pH 6.5)) containing ^{3}H-labeled and unlabeled biotin and tracer amount of [^{14}C]inulin (as an extracellular marker to correct for adhering extracellular medium). The incubation solution was added to the confluent Caco-2 monolayers at the onset of experiment. At the end of the incubation period, the incubation media were removed by aspiration. The monolayers were immediately washed twice with ice-cold buffer then digested with 0.5 ml of 1 N NaOH for 1 h at 70°C, neutralized with HCl then counted for radioactivity (after adding scintillation fluid) using a Beckman liquid scintillation counter. Protein concentrations of cells were measured on parallel wells by the method of Lowry et al. [15] using bovine serum albumin as the standard. Results are presented as mean ± S.E. of separate monolayers and are expressed as pmol/mg protein per unit time. Uptake kinetic parameters (i.e., the V_{max} and the apparent K_m) were calculated using a computerized model of the Michaelis-Menten equation as described previously by Wilkinson [16].

Results

Time-course and general characteristics of biotin uptake

Uptake of low (4 nM) and high (20 μM) concentrations of biotin by confluent monolayers of Caco-2 cells was appreciable and linear for 10 min of incubation and occurred at a rate of 0.51 pmol/mg protein per min and 1215 pmol/mg protein per min, respectively (Fig. 1A,B). Extending the uptake line to zero time intercepted the y-axis at 0.103 and 1592 pmol/mg protein for 4 nM and 20 μM biotin, respectively, indicating minimal substrate binding to cell surface. Based on these studies, 3 min incubation time was adopted as the standard incubation period in all subsequent studies.

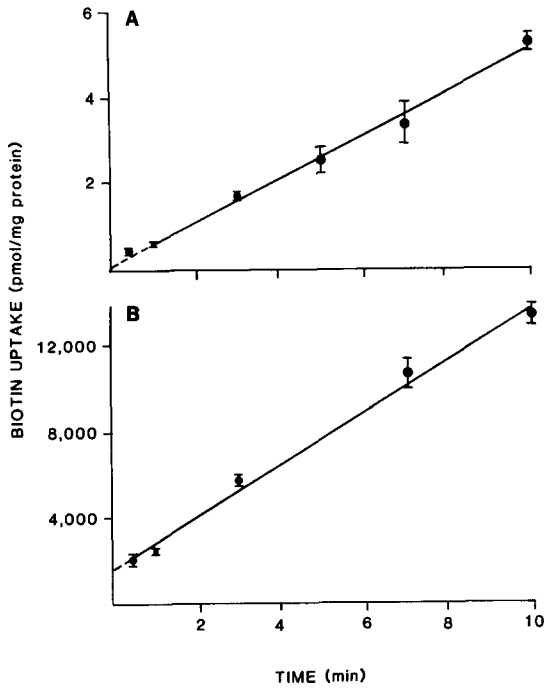

Fig. 1. Uptake of biotin by confluent Caco-2 cells as a function of time. Confluent Caco-2 cells were incubated at 37°C with Krebs-Ringer phosphate buffer (pH 6.5) in the presence of 4 nM (A) and 20 μM (B) biotin. Each data point is the mean ± S.E. of three or four separate transport determinations. For (A) $Y = 0.52X + 0.103$, $r = 0.99$. For (B) $Y = 1.22X + 1.59$, $r = 0.99$.

Transmembrane ion gradients play a vital role in substrate transport via membrane-linked energy transduction. Two ions, Na^+ and H^+, have been identified as the main coupling ions in such transport processes. For this reason, we examined the effect of replacing the Na^+ gradient isoosmotically with a gradient of other monovalent cations, namely K^+, choline, Li^+ and NH_4^+ (i.e., reducing the Na^+ concentration in the incubation medium from 143 mM to 20 mM) on the uptake of biotin (4 nM) by confluent Caco-2 cells. The results showed significant inhibition of biotin uptake upon replacing the Na^+ gradient, regardless of the cation used to replace it (Table I). The uptake of biotin in the presence of Li^+, however, was comparatively higher than when K^+, choline or NH_4^+ was used, indicating that Li^+ could partially substitute for Na^+ in supporting biotin uptake.

The effect of varying the pH of Na^+-containing incubation media (i.e., varying the H^+ concentration) on biotin uptake by confluent Caco-2 cells was also examined. The results (Table II) showed only minimal increase in biotin uptake upon lowering the pH of the incubation media from 8 to 5. Similarly, biotin uptake in the absence of Na^+ increased slightly with decreasing incubation buffer pH (uptake of 0.38 ± 0.06, 0.41 ± 0.02, 0.51 ± 0.04 and 0.52 ± 0.02 pmol/mg protein per 3 min at buffer pH 8, 7, 6 and 5, respectively.

TABLE I

Effect of Na^+ and other cations on biotin uptake by confluent Caco-2 cells

Confluent Caco-2 cells (7–10 days post-confluence) were incubated for 3 min (initial rate) at 37°C with Krebs-Ringer phosphate buffer (pH 6.5). The Na^+ concentration of the control incubation media was 143 mM. As indicated above, Na^+ concentration was reduced to 20 mM and replaced isoosmotically by other cations. Biotin (4 nM) was added to the incubation medium at the onset of incubation.

Condition	Uptake (pmol/mg protein per 3 min)	P value [a]
143 mM Na^+ (Control)	1.83 ± 0.09 (9) [b]	
20 mM Na^+ + 123 mM K^+	0.63 ± 0.06 (6)	< 0.01
20 mM Na^+ + 123 mM choline	0.67 ± 0.03 (9)	< 0.01
20 mM Na^+ + 123 mM NH_4^+	0.74 + 0.07 (3)	< 0.01
20 mM Na^+ + 123 mM Li^+	1.10 ± 0.07 (6)	< 0.01

[a] P values were calculated using the Student's t-test; comparison was made relative to simultaneously performed controls.
[b] Number of separate transport determinations.

We also examined the effect of incubation temperature on biotin uptake. Uptake of 4 nM biotin was found to be significantly ($P < 0.01$) higher in confluent Caco-2 cells incubated at 37°C than at 4°C (2.11 ± 0.04 ($n = 3$) and 0.65 ± 0.08 ($n = 3$) pmol/mg protein per 3 min, respectively). In another study, we determined whether metabolic alterations occur in the biotin molecule during its uptake by confluent Caco-2 cells. This was done using silica gel pre-coated HPTLC and a solvent system of benzene/methanol/acetone/anhydrous acetic acid (70:20:5:5, v/v). In this study, the confluent Caco-2 cells were incubated with 16 nM [^{3}H]biotin for 3 min. The results indicated that the majority (93.3%) of the radioactivity taken up by these cells was in the form of intact biotin.

TABLE II

Effect of incubation buffer pH on biotin uptake by confluent Caco-2 cells

The confluent Caco-2 cells were incubated for 3 min at 37°C with Krebs-Ringer buffer. The buffering system used in this study was 10 mM Hepes and 10 mM Mes instead of NaH_2PO_4; thus the final Na^+ concentration in the incubation media was 133 mM. Biotin (4 nM) was added to the incubation medium at the onset of incubation.

Buffer pH	Uptake (pmol/mg protein per 3 min)
5.0	1.89 + 0.11 (3) [a]
5.5	1.81 + 0.08 (3)
6.0	1.73 + 0.03 (3)
6.5	1.83 + 0.05 (3)
7.0	1.78 + 0.05 (3)
7.5	1.67 + 0.05 (3)
8.0	1.67 + 0.02 (3)

[a] Number of separate transport determinations.

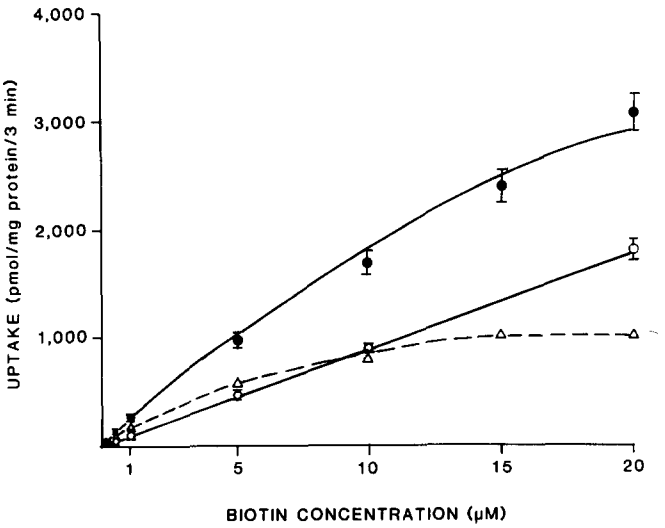

Fig. 2. Uptake of biotin by confluent Caco-2 cells as a function of biotin concentration in the incubation media. Confluent Caco-2 cells were incubated for 3 min (initial rate) with Krebs-Ringer phosphate buffer (pH 6.5) at 37°C (closed circles) and 4°C (opend circles) in the presence of different concentrations of biotin. Data are means ± S.E. of three or four separate transport determinations. Dotted line represents uptake by the temperature-dependent process.

Uptake as a function of biotin concentration

Fig. 2 shows the initial rate of biotin uptake by confluent Caco-2 cells as a function of increasing substrate concentration in the incubation media. The study was carried out at both 37°C and 4°C. At 37°C, uptake of biotin was found to include a saturable component. At 4°C, the uptake was lower and linear. Uptake of biotin by the temperature-dependent saturable process was calculated by subtracting uptake at 37°C from uptake at 4°C. The kinetic parameters were determined by the non-linear method described in Materials and Methods, and found to be 520 pmol/mg protein per min for the V_{max} and 9.5 μM for the apparent K_m.

Effect of unlabeled biotin and structural analogues

The effect of different concentrations (25 and 50 μM) of unlabeled biotin and the structurally related compounds desthiobiotin, biocytin, biotin methyl ester and diaminobiotin on the uptake of [^{3}H]biotin (4 nM) by confluent Caco-2 cells was examined at 37°C. Unlabeled biotin and desthiobiotin caused significant inhibition ($P < 0.01$) of biotin uptake (Table III). When the inhibitory effect of the structural analogue desthiobiotin was further analyzed by the Dixon method, a competitive-type inhibition was found with an inhibition constant (K_i) of 41 μM (Fig. 3). In contrast, biocytin was found to be a weak inhibitor of biotin uptake while biotin methyl ester and diaminobiotin had no effect (Table III).

When the effect of 50 μM unlabeled biotin on the uptake of 4 nM [^{3}H]biotin was examined at 4°C or in choline buffer (no Na^+), no further inhibition of [^{3}H]biotin uptake was seen above that already caused by these conditions individually (uptake of 2.11 ± 0.04 ($n = 4$), 0.65 ± 0.08 ($n = 3$), 0.58 ± 0.09 ($n = 3$), 0.65 ± 0.08 ($n = 4$), 0.62 ± 0.09 ($n = 4$) pmol/mg protein per 3 min at 37°C in Na^+ buffer (control), 37°C in choline buffer, 37°C in choline buffer containing unlabeled biotin, 4°C in Na^+ buffer and 4°C in Na^+ buffer containing unlabeled biotin, respectively).

TABLE III

Effect of unlabeled biotin and structural analogues on the uptake of [^{3}H]biotin by confluent Caco-2 cells

The confluent Caco-2 cells were incubated for 3 min at 37°C with Krebs-Ringer phosphate buffer (pH 6.50 in the presence of 4 nM [^{3}H]biotin and the compound under investigation. Results are means ± S.E.

Compound	Concn. (μM)	Uptake (pmol/mg protein per 3 min)	P value [a]
Control		1.69 ± 0.04 (3) [b]	
Unlabeled biotin	25	0.664 ± 0.03 (3)	< 0.01
	50	0.56 ± 0.02 (3)	< 0.01
Desthiobiotin	25	0.95 ± 0.02 (3)	< 0.01
	50	0.73 ± 0.01 (3)	< 0.01
Biocytin	25	1.47 ± 0.12 (3)	
	50	1.16 ± 0.05 (3)	< 0.01
Biotin methyl ester	25	1.52 ± 0.04 (3)	
	50	1.69 ± 0.07 (3)	
Diaminobiotin	25	1.80 ± 0.02 (3)	
	50	1.60 ± 0.10 (3)	

[a] P values were calculated using the Student's t-test; comparison was made relative to simultaneously performed controls.
[b] Number of separate transport determinations.

Effect of gramicidin, valinomycin and ouabain

Table IV depicts the effect of pretreating confluent Caco-2 cells with the monovalent cation ionophore gramicidin (30 μg/ml), the K^+-ionophore valinomycin (30 μg/ml) and the Na^+,K^+-ATPase inhibitor ouabain

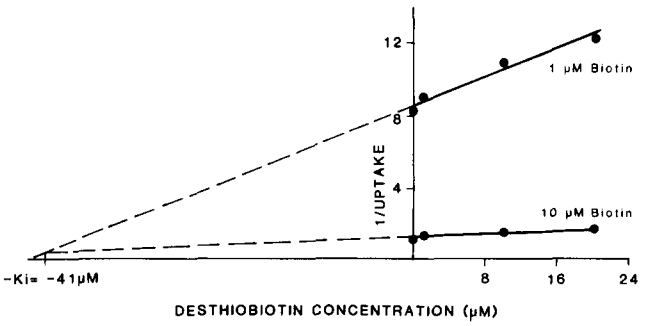

Fig. 3. Dixon plot of the effect of desthiobiotin on the uptake of [^{3}H]biotin by confluent Caco-2 cells. Confluent Caco-2 cells were incubated for 3 min (initial rate) at 37°C with Krebs-Ringer phosphate buffer (pH 6.5), in the presence of 1 and 10 μM [^{3}H]biotin and different concentrations of desthiobiotin. Each data point was derived from separate experiments.

TABLE IV

Effect of gramicidin, valinomycin and ouabain on biotin uptake by confluent Caco-2 cells

The confluent Caco-2 cells were pretreated at 37°C with Krebs-Ringer phosphate buffer containing the compound under investigation, washed, then incubated at 37°C in the presence of 4 nM [^{3}H]biotin. Uptake was then performed for 3 min. In (A) gramicidin and valinomycin were delivered in ethanol (0.5%) and identical amount of ethanol was added to control.

	Uptake (pmol/mg protein per 3 min)	*P* value [a]
(A) Control	1.40 ± 0.02 (4) [b]	
Gramicidin (30 μg/ml)	0.87 ± 0.06 (4)	< 0.01
Valinomycin (30 μg/ml)	1.40 ± 0.8 (4)	
(B) Control	1.54 ± 0.06 (4)	
Ouabain 10 mM	1.01 ± 0.08 (4)	< 0.01

[a] *P* values were calculated using the Student's *t*-test; comparison was made relative to simultaneously performed controls.

[b] Number of separate transport determinations.

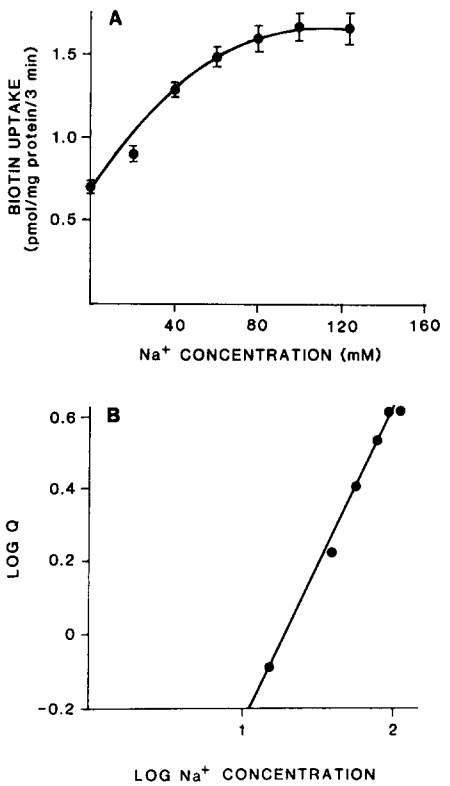

Fig. 4. (A) Biotin uptake by confluent Caco-2 cells as a function of Na^+ concentration in the incubation media. Confluent Caco-2 cells were incubated for 3 min (initial rate) with modified Krebs-Ringer buffer (pH 6.5) at 37°C containing different concentrations of Na^+ (the concentration of NaCl was varied and replaced isoosmotically with choline chloride) and a buffering system of 40 mM Hepes (no NaH_2PO_4). Valinomycin (which has no effect on biotin uptake, see Results) was added to the incubation medium at 30 μg/ml. Each data point is mean ± S.E. of three or four separate transport determinations. (B) The Hill plot of the data in A.

(10 mM) on the initial rate of biotin (4 nM) uptake. Significant ($P < 0.01$) inhibition of biotin uptake was seen with gramicidin and ouabain but not with valinomycin.

Stoichiometry of the biotin–Na^+ coupling

In this study, we examined the stoichiometry of the biotin–Na^+ coupling using the 'Activation Method' as described previously [17–19]. This was done by examining the initial rate of biotin (4 nM) uptake by confluent Caco-2 cells as a function of Na^+ concentration in the incubation media. Biotin uptake was found to be saturable as a function of Na^+ concentration (Fig. 4A). When the data in Fig. 4A was applied to the Hill plot (Fig. 4B), a linear ($r = 0.99$) relationship was found with a Hill coefficient of 0.97, indicating an Na^+ to biotin coupling ratio of 1:1.

Regulation of biotin uptake by the substrate level: effect of biotin deficiency

Adaptive regulation of biotin uptake by the substrate level in the growth media was investigated in these studies. This was done by examining the effect of growing Caco-2 monolayers in a biotin-deficient media on the kinetic parameters of the substrate uptake. Growing cells in a biotin-deficient media resulted in a marked (258%) increase in the V_{max} of biotin uptake compared to cells grown simultaneously under biotin sufficient condition, i.e., grown in normal growth media (3230 and 1250 pmol/mg protein per 3 min, respectively). On the other hand, no change in the apparent K_m of the uptake system was seen between the deficient and control cells (7.50 and 6.89 μM, respectively).

Discussion

The aim of the present study was to investigate the mechanism of biotin uptake of Caco-2 and examine the suitability of Caco-2 cells as an in vitro cell culture system for studying the cellular mechanism and regulation of biotin uptake. We chose Caco-2 cells because they are of human origin and because of the recent findings which demonstrate that these cells possess many of the transport characteristics of the native small intestinal absorptive cells [10–13]. These cells when confluent undergo enterocyte-like differentiation and polarization with formation of brush border microvilli on the apical surface and tight junctions between adjacent cells [7–9]. The well developed tight junctions of confluent monolayers of Caco-2 cells allow separate compartmentalization of apical and basolateral membranes [7–13]. Therefore, by introducing a nutrient to apical or basolateral surface, transport across that membrane domain can be selectively studied [10–13].

Our results showed that biotin uptake by these cells was appreciable and was linear with time for up to 10 min of incubation both at low and high biotin concentrations. The initial rate of uptake of biotin was strongly dependent on the Na^+ gradient in the incubation media. This conclusion was based on the observations that substituting Na^+ gradient isoosmotically with a gradient of other monovalent cations like K^+, choline, Li^+, and NH_4^+ caused a severe inhibition of biotin uptake. Interestingly, however, substituting Na^+ by Li^+ caused a relatively lesser inhibition of biotin uptake than when other cations were used suggesting that Li^+ could partially substitute for Na^+ in stimulating biotin uptake. The conclusion on the effect of Na^+ on biotin uptake was further confirmed by the observations that pretreatment of Caco-2 monolayers with gramicidin (an ionophore for monovalent cations which causes dissipation in the inwardly directed cation (Na^+) gradient) and ouabain (an inhibitor of Na^+,K^+-ATPase, the enzyme that maintains the inwardly directed Na^+ gradient by continuously pumping Na^+ out of the cell) caused a significant inhibition in the initial rate of biotin uptake. In contrast to the enhancing effect of Na^+ on biotin uptake, varying the H^+ concentration (i.e., pH) of the incubation medium only slightly affected biotin uptake.

To test for the existence of a carrier-mediated system for biotin uptake in this intestinal cell line, we examined the initial rate of the vitamin uptake as a function of concentration at 37°C and 4°C. A temperature-dependent saturable component was found to exist with a V_{max} of 520 pmol/mg protein per min and an apparent K_m of 9.5 μM. These findings indicated that biotin uptake across the apical membrane of Caco-2 cells was through a carrier-mediated process.

The existence of a carrier system for biotin uptake in Caco-2 cells was further confirmed in the present study by the findings that unlabeled biotin and the structural analogue desthiobiotin, when added to the incubation medium at physiological temperature (37°C), caused a significant inhibition in the initial rate of [^{3}H]biotin uptake. A similar biotin carrier system has been demonstrated in animal and human small intestine [2–6]. The inhibitory effect of desthiobiotin was found to be competitive in nature as indicated by the finding with the Dixon plot (Fig. 3) with an inhibition constant (K_i) of 41 μM. It is interesting to mention here that unlabeled biotin did not cause any further inhibition of [^{3}H]biotin uptake by Caco-2 cells when incubated at 4°C. Similarly, in the absence of Na^+ in the incubation media, unlabeled biotin did not cause further inhibition of [^{3}H]biotin uptake. These findings, together with the finding shown in Fig. 2 (demonstration of lack of saturation of biotin uptake at 4°C), indicate that the uptake process of biotin under these conditions is via a non-carrier mediated process.

The study with the structural analogues also provided interesting information concerning the structure–activity relationship between the biotin molecule and its carrier system. Significant inhibition of [^{3}H]biotin uptake was seen with unlabeled biotin and desthiobiotin compared to biocytin and biotin methyl ester (compounds in which the carboxyl group of the valeric acid moiety is blocked) and diaminobiotin (a compound that lacks an intact carbamide moiety). These findings suggest that a free carboxyl group and an intact carbamide moiety in the imidazolidone ring of the biotin molecule are important for efficient interaction with the carrier system. The ability of desthiobiotin (a compound which lacks the sulfur moiety in the tetrahydrothiopene ring of the biotin molecule) to significantly inhibit H-biotin uptake also suggests that the sulfur moiety in the tetrahydrothiopene ring is likely to be less important in the interaction of the substrate with the carrier system. Our present findings are in direct contrast to previous study by Cogburn et al. [14] in which these investigators demonstrated absence of carrier mediated transport for biotin. The cause of this discrepancy is not clear but could be due to the fact that Cogburn et al. [14] have used higher working biotin concentrations (10, 100, 1000 and 10000 μM) to investigate the existence of a carrier system, a concentration range above the capacity of the biotin carrier of these cells.

Pretreatment of cells with the K^+-ionophore valinomycin has been shown to induce significant changes in transmembrane electrical potential [20]. When confluent Caco-2 cells were pretreated with valinomycin, no significant effect on biotin uptake was found. These findings suggested that the uptake process of the anionic biotin (pK_a of biotin = 4.65) by confluent Caco-2 cells across the apical membrane is electroneutral in nature. This suggestion was further supported by the finding in the stoichiometric study (Fig. 4A and B) in which a Hill coefficient of 0.97 was obtained, indicating a coupling ratio between biotin$^-$ and Na^+ of 1:1.

Following the elucidation of the biotin uptake system in confluent Caco-2 cells, we investigated possible regulation of this system by the substrate level in the culture media. For this purpose, the confluent Caco-2 cells were grown in a biotin-deficient media and the kinetic parameters of the biotin uptake process was examined. Cells grown in biotin-deficient media had a marked increase in biotin uptake compared to those grown in biotin-sufficient (normal) media, suggesting up-regulation of biotin carrier. While there was a marked increase in V_{max} (258%) induced by biotin deficient media, there was no difference in the apparent K_m of the uptake system between the cells incubated with biotin-deficient or normal media. These findings demonstrate the ability of this human-derived intestinal epithelial cells to rapidly up-regulate its bi-

otin uptake process in biotin-deficient environment. Furthermore, these findings suggest that the regulation is mediated via changes in the number and/or activity (V_{max}) of the biotin uptake carriers rather than a change in their affinity (K_m). This is the first reported demonstration of such up-regulation of biotin uptake in human-derived intestinal cells. A previous study demonstrated that when rats were fed biotin-deficient diet for 5–6 weeks, biotin-deficient rats developed signs and symptoms of biotin deficiency [6]. The intestinal brush border membrane uptake of these biotin-deficient rats were similarly markedly increased. The V_{max} of intestinal brush border membrane uptake of biotin-deficient rat was increased (146–230%) with minimal change in K_m. Our present findings with Caco-2 cells suggest that the up-regulation of biotin uptake by the intestinal cells is likely to occur relatively quickly during biotin deficiency states and is regulated at the cellular level. The reversibility and the exact time frame of this regulatory change and the intracellular level at which the change is happening (transcriptional and/or translational) are not known and require further investigations.

The present findings on biotin uptake by confluent Caco-2 cells are very similar to and further validate some of the findings previously described in human and animal small intestine [1–6], and dispute other findings [14] previously reported for Caco-2 cells. In summary, these studies demonstrate presence of specialized biotin carrier system in Caco-2 cells. The dependency of this uptake system on Na^+ and the stoichiometric ratio of biotin$^-$-Na^+ coupling, the structural requirements of the biotin molecule that are needed for proper interaction with the uptake carrier, and the electrical nature of the uptake processes are similar to human and animal small intestine. These studies also show the ability of Caco-2 cells to rapidly up-regulate biotin uptake in a biotin-deficient environment. Thus, our present findings demonstrate and validate the relevance and suitability of this human-derived intestinal cell line as an in vitro model system to study the finer details of cellular and molecular regulation of biotin intestinal uptake.

Addendum

After preparing and submitting the manuscript, we have performed a new study in which we examined possible expression of the Caco-2 biotin uptake system in *Xenopus laevis* oocytes following injection of oocytes with poly(A)$^+$ mRNA of Caco-2 cells. The study was done using the *Xenopus* oocyte system as described previously [22–26]. Briefly, stage V–VI *Xenopus* oocytes were defolliculated manually after treatment with 1.5 mg/ml collagenase (Type A, Boehringer-Mannheim) in Ca^{2+}-free modified Barth's solution (NaCl 82 mM; KCl 2 mM; $MgCl_2$ 1 mM; Hepes 5 mM). Defolliculated cells were maintained at 14°C in Barth's solution (NaCl 88 mM; KCl 2 mM; $NaHCO_3$ 2.4 mM; $MgSO_4$ 0.82 mM; $Ca(NO_3)_2$ 0.33 mM; $CaCl_2$ 0.41 mM; Hepes 10 mM (pH 7.4)) containing 50 μg/ml gentamicin. Healthy oocytes were selected 18 h later and injected with 50 nl (1.0 ng/nl) or poly(A)$^+$ mRNA isolated from Caco-2 cells by standard procedures [21] and double-selected on oligo-d(T)-cellulose. Control oocytes were injected with the same amount of water. Oocytes were incubated at 14°C for 3–4 days in Barth's solution, with daily changes of medium, and removal of unhealthy cells. Oocytes were then used to assay [^{3}H]biotin uptake by incubating cells for 2 h in Ringer's solution (NaCl 120 mM; KCl 2 mM; $CaCl_2$ 1.8 mM; Hepes 5 mM) at room temperature. Expression of the D-glucose transport system in mRNA-injected oocytes was used as an internal control for mRNA quality, since expression of this system in oocytes has been well established and documented [22,23]. Oocytes injected with mRNA demonstrated an average of 7.4 ± 2 ($n = 6$) fold increase in D-glucose (30 nM) uptake over water-injected oocytes. [^{3}H]Biotin (23 nM) uptake in mRNA-injected oocytes was found to be significantly induced ($P < 0.01$) compared to water-injected oocytes by an average of 6.3 ± 1.5 fold ($n = 16$), consistent over several different donors.

These results further support the existence of a carrier-mediated system for biotin uptake in Caco-2 cells, and demonstrate the suitability of using *Xenopus* oocytes in expression cloning studies of the biotin transport carrier.

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