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The efflux of biotin from human peripheral blood mononuclear cells

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Peripheral blood mononuclear cells (PMBCs) are readily available for sampling and are a useful model for studying biotin metabolism in human cells. To better understand biotin handling by PMBCs, we investigated the mechanism(s) and kinetics of biotin efflux from PMBCs. Human PMBCs were incubated with [^{3}H]biotin at 475 pmol/L to load the cells. The [^{3}H]biotin-loaded cells were then harvested and incubated in [^{3}H]biotin-free media for up to 20 hours. At various intervals, aliquots of the PMBC suspensions were collected and analyzed for intracellular [^{3}H]biotin. [^{3}H]Biotin efflux from cells at 37°C was fast and triphasic; the half-lives for the three elimination phases were 0.2 ± 0.02 hours, 1.2 ± 0.1 hours, and 21.9 ± 13.6 hours. Such a triphasic [^{3}H]biotin efflux could reflect (1) rapid efflux of free biotin, (2) slower release of biotin bound to intracellular molecules, and (3) even slower release from carboxylases in cellular organelles. Incubation at 4°C rather than 37°C increased the [^{3}H]biotin retained at 20 hours from 27% to 85%. This observation is consistent with transporter-mediated efflux. When cellular glucose utilization was reduced by 2-deoxy-D-glucose and sodium fluoride, [^{3}H]biotin efflux was similar to controls, suggesting that biotin efflux does not directly require metabolic energy. When [^{3}H]biotin-loaded cells were incubated in external medium containing unlabeled biotin analogs, [^{3}H]biotin efflux was accelerated approximately two times compared with incubation in a biotin-free medium. This observation suggests that biotin efflux is mediated by the same transporter that mediates biotin uptake from the extracellular medium (i.e., classic countertransport). (J. Nutr. Biochem. 10:105–109, 1999)

Keywords: biotin; efflux; half-lives; peripheral blood mononuclear cells

Introduction

The uptake of biotin into various cell types and tissues has been extensively studied and recently reviewed.[1] The uptake and metabolism of biotin in human peripheral blood mononuclear cells (PMBCs) is particularly interesting because, unlike red cells, PMBCs are readily available for sampling and have intact mitochondria and biotin-dependent carboxylases. The uptake and metabolism of biotin in PMBCs have been reported recently.[2] The uptake of [^{3}H]biotin into PMBCs is mediated by an energy-requiring transporter that depends on Na-K-ATPase. PMBCs do not appear to catabolize biotin (in contrast to hepatocytes) and hence the [^{3}H] in PMBCs quantitates [^{3}H]biotin rather than [^{3}H]biotin metabolites.

Usually the cellular uptake of compounds is quantitated as net uptake; that is, the sum of two opposing processes: transport into the cells and release from the cells into the medium. In most studies, changes in the rate of the net cellular uptake of a given compound are attributed to a change in the rate of transport into cells. However, to thoroughly understand net uptake, changes in the rate of release also must be considered. For essential nutrients such as vitamins, cellular release may have a strong impact on the delivery of nutrients from blood to tissues, as exemplified by vitamin B6 delivery to tissues by erythrocytes.[3] In the present study, we sought to further refine our understanding of biotin transport by PMBCs by investigating the rate and the mechanism of biotin release.

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Methods and materials

We combined pharmacokinetic and biochemical methods to study the release of biotin from cells. (1) Pharmacokinetic analysis of the concentration-versus-time curves of biotin in cells was used to identify compartments of biotin distribution and the rate of biotin elimination from cells. (2) Biochemical methods were used to investigate the mechanism by which biotin is released from the cells into the medium.

Isolation of PMBCs

Peripheral blood was collected from three male and two female Caucasians. PMBCs were isolated from blood by gradient centrifugation as described previously.[2,4] Cells from various subjects were not pooled. Viability of PMBCs was measured by exclusion of 4.16 mmol/L Trypan blue and was 99.4 ± 0.6%; viability did not change during the incubations unless noted otherwise.

PMBCs incubations

Rates of [^{3}H]biotin efflux as a function of temperature and glucose utilization. The PMBC pellet isolated from 100 mL of blood as described above was resuspended in 25 mL of phosphate buffered saline (PBS; 1.47 mmol/L KH_2PO_4, 2.68 mmol/L KCl, 8.10 mmol/L K_2HPO_4, 136.89 mmol/L NaCl, pH 7.4); this medium produced an inwardly directed sodium gradient and contained 2.75% bovine serum albumin and 5.5 mmol/L D-glucose to maintain cell viability. To load the PMBCs with [^{3}H]biotin, the suspension was warmed at 37°C for approximately 15 minutes and sufficient [^{3}H]biotin was added to produce a final concentration of 475 pmol/L (Dupont, Boston, MA USA; specific radioactivity 2.15 TBq/mmol); the tritium label is located on the α and β carbons to the carboxyl group of the biotin side chain. PMBCs were incubated in the [^{3}H]biotin medium for a total of 2 hours at 37°C; triplicate 0.5-mL aliquots were collected at 0.25, 0.5, 1.0, 1.5, and 2.0 hours. Each aliquot was immediately centrifuged at 2,260 g for 100 seconds. The supernatants were discarded, and each PMBC pellet was resuspended in PBS and washed three times to remove extracellular [^{3}H]biotin. Each washed PMBC pellet was suspended in 0.4 mL of PBS and transferred from the incubation vials into scintillation vials containing 0.5 mL of 0.5% Triton X-100. The incubation vials were washed two times with 0.4 mL saline and the washings were added to the scintillation vials. Liquid scintillation fluid (4.5 mL) was added (Ultima Gold XR, Packard Instrument Company, Meriden, CT USA) and [^{3}H] was quantitated in a liquid scintillation analyzer Tri-Carb 1900-TR (Packard). Previously, we showed that PMBCs do not catabolize biotin during incubations up to 168 hours.[2] Thus, [^{3}H] in PMBCs quantitates [^{3}H]biotin per se rather than [^{3}H]biotin plus metabolites.

[^{3}H]Biotin efflux from PMBCs was quantitated as original [^{3}H] after the 2-hour loading minus [^{3}H] that remained in the PMBCs at a given time up to 20 hours as follows. After 2 hours, the PMBC suspension (volume = 17.5 mL) was centrifuged at 250 g for 10 minutes. The supernatant was discarded and the PMBC pellet was suspended in 17.5 mL of [^{3}H]biotin-free medium. This medium contained 2.75% bovine serum albumin and 5.5 mmol/L D-glucose in PBS and is denoted as control. To study the effect of glucose utilization on [^{3}H]biotin efflux, D-glucose in the medium was replaced by 1 mmol/L sodium fluoride plus 5.5 mmol/L 2-deoxy-D-glucose. To study effects of temperature, PMBCs were incubated in biotin-free medium for 20 hours at either 4°C or 37°C; 0.5-mL aliquots were collected in triplicate at 0.1, 0.15, 0.2, 0.5, 0.75, 1.0, 2.0, 4.0, and 20.0 hours. The samples were analyzed for [^{3}H]biotin as described above. PMBCs from the same five subjects were used for each experiment.

[^{3}H]Biotin efflux in the presence of the inhibitors phloretin and 4,4′-diisothiocyanatostilbene-2,2′-D,L-sulfonic acid (DITS). PMBCs were loaded with [^{3}H]biotin in standard medium for 2 hours as described above; loading was confirmed as described above. [^{3}H]Biotin efflux in the presence of either 200 μmol/L phloretin or 200 μmol/L DITS in the biotin-free medium was quantitated; controls were incubated in medium without inhibitor.

[^{3}H]Biotin efflux in the presence of iodoacetate. To study the effect of the sulfhydryl-group modifying agent iodoacetate, PMBCs were loaded with [^{3}H]biotin for 2 hours as described above, with one modification: Albumin was not included in the medium to avoid reaction of albumin with iodoacetate. After 2 hours of loading, six 1-mL aliquots were analyzed for intracellular [^{3}H]biotin as described above. To measure [^{3}H]biotin efflux, the remaining PMBC suspension was centrifuged for 10 minutes at 250 g. The supernatant was discarded and the PMBC pellet was suspended in a volume of biotin-free medium equal to the supernatant volume. Iodoacetate was added to produce a concentration of 1 mmol/L; controls did not contain iodoacetate. PMBCs were incubated at 37°C for 30 minutes, and six 1-mL aliquots were analyzed for intracellular [^{3}H]biotin as described above.

[^{3}H]Biotin efflux in the presence of unlabeled biotin or biotin analogs. PMBCs were loaded with [^{3}H]biotin for 2 hours as described above. After 2 hours of loading, six 0.5-mL aliquots were collected and analyzed for intracellular [^{3}H]biotin. To measure [^{3}H]biotin efflux, the remaining PMBC suspension was centrifuged for 10 minutes at 250 g, the supernatant was discarded, and the PMBC pellet was resuspended in the original volume of biotin-free medium, which contained now either unlabeled biotin, 2-iminobiotin, diaminobiotin, or D,L-desthiobiotin (all at 1 mmol/L); control medium contained neither biotin nor analogs. After incubating at 37°C for 30 minutes, six 0.5-mL aliquots were collected from each experiment and analyzed for [^{3}H]biotin. Thirty-minute rather than 20-hour incubations were chosen because more rapid efflux of [^{3}H]biotin in the presence of extracellular biotin or biotin analogs leaves little remaining [^{3}H]biotin by 20 hours.

Pharmacokinetic calculations

Using the concentration-versus-time curves of [^{3}H]biotin in PMBCs in the [^{3}H]biotin-free control medium at 37°C, the disposition half-lives of [^{3}H]biotin were calculated by nonlinear regression.[5] Correlation coefficients of the regression lines and log transformation of the data suggested that a three-compartment model (Eq. 1) was the most appropriate model to fit the data. Kaleidagraph 3.0.5 was used for nonlinear regression analysis (Abelbeck Software, Reading, PA USA). The disposition rate constants as obtained by the use of equation 1 were transformed into disposition half-lives (Eq. 2).

$$C_t = C_\alpha * e^{-k\alpha * t} + C_\beta * e^{-k\beta * t} + C_\gamma * e^{-k\gamma * t} \quad (1)$$

where C_t (amol/10^6 cells) is biotin concentration at time t; t (h) is time; C_α, C_β, or C_γ (amol/10^6 cells) are zero-time intercepts of the biotin concentrations for the individual phases of distribution; e is 2.718; k_α, k_β, and k_γ (h^{-1}) are the disposition rate constants during the phases of distribution.

$$t_{(1/2)\alpha,\beta\gamma} = \ln 2/k_{\alpha,\beta\gamma} \quad (2)$$

where $t_{(1/2)\alpha,\beta,\gamma}$ (h) is disposition half-life of biotin during the α, β, or γ phases of disposition; ln 2 is 0.693; and $k_{\alpha,\beta,\gamma}$ (h^{-1}) is the disposition rate constant during the α, β, or γ phases of disposition.

The areas under the [^{3}H]biotin concentration-versus-time curves were calculated using the linear trapezoidal rule.[5]

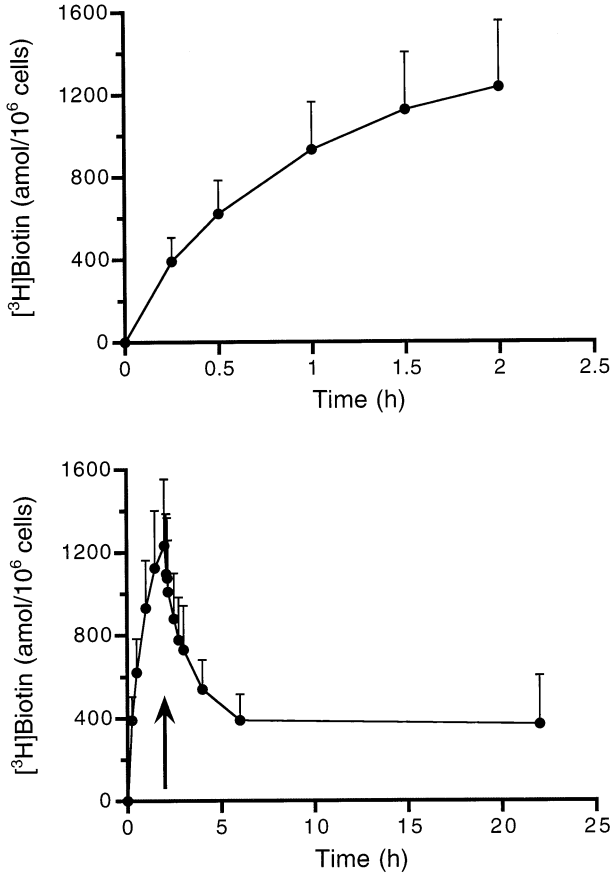

Figure 1 *Upper panel:* Uptake of [^{3}H]biotin into peripheral blood mononuclear cells (PMBCs). PMBCs were incubated in phosphate buffered saline containing 2.75% bovine serum albumin, 5.5 mmol/L D-glucose, and 475 pmol/L [^{3}H]biotin at 37°C for 2 hours (values are means ± SD from five subjects; PMBCs from each subject were assayed in triplicate). *Lower panel:* Efflux of [^{3}H]biotin from PMBCs. PMBCs were loaded with [^{3}H]biotin for 2 hours (same as in upper panel); the [^{3}H]-loaded PMBCs were transferred into [^{3}H]biotin-free medium and incubation was continued for 20 hours at 37°C. The arrow indicates the time when the PMBCs were transferred into [^{3}H]biotin-free medium (values are means ± SD from five subjects; PMBCs from each subject were assayed in triplicate).

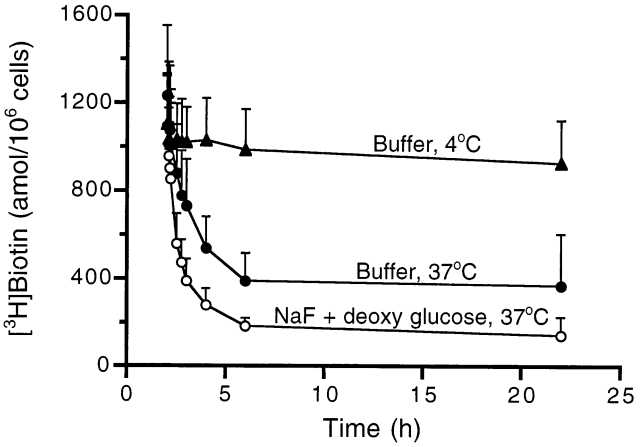

Figure 2 The efflux of [^{3}H]biotin from peripheral blood mononuclear cells (PMBCs) into the medium as a function of temperature and glucose utilization. PMBCs were incubated in phosphate buffered saline (PBS) containing 2.75% bovine serum albumin, 5.5 mmol/L D-glucose, and 475 pmol/L [^{3}H]biotin at 37°C for 2 hours. Then the [^{3}H]-loaded PMBCs were transferred into [^{3}H]biotin-free PBS, containing 2.75% bovine serum albumin, and 5.5 mmol/L D-glucose at either 4°C or 37°C, or into biotin-free PBS containing 2.75% bovine serum albumin plus 1 mmol/L sodium fluoride and 5.5 mmol/L 2-deoxy-D-glucose at 37°C; incubations were continued for 20 hours. For clarity, data points during the loading phase with [^{3}H]biotin are not shown (values are means ± SD from the same five subjects in the three experiments; PMBCs from each subject were assayed in triplicate).

Statistics

Significance of differences among groups was tested by one-way analysis of variance (ANOVA) using SuperANOVA 1.11 (Abacus Concepts, Inc., Berkeley, CA USA). Dunnett's post-hoc procedure was used for posthoc testing; the Dunnett procedure compares the control mean to the means for each treatment group.[6] Differences were considered significant if the *P*-value was less than 0.05. Means ± 1 SD are reported.

Results

When PMBCs were incubated in [^{3}H]biotin-containing medium at 37°C for 2 hours, the cells accumulated [^{3}H]biotin; [^{3}H]biotin concentrations approached a steady-state (*Figure 1*, upper panel). In our previous studies, we demonstrated that the [^{3}H] detected in the cell pellet was truly intracellular [^{3}H]biotin rather than biotin adsorbed to cell surfaces or incubation vials or transformed into biotin metabolites.[2] True transport into the cell (rather than absorption to surfaces) was confirmed here by the observation that incubation of [^{3}H]biotin with PMBCs at 4°C and subsequent washing of the cells with cold PBS reduced [^{3}H]biotin uptake by 99.7% compared with transport at 37°C.

After transfer into [^{3}H]biotin-free medium, the efflux rate of [^{3}H]biotin from PMBCs was measured in [^{3}H]biotin-loaded PMBCs. At 37°C, the cells released biotin rapidly (*Figure 1*, lower panel); after 20 hours in control medium, only 27 ± 12% of [^{3}H] remained in the PMBCs. The efflux curves of [^{3}H]biotin are accurately fit ($r = 0.997 \pm 0.002$) by a tri-exponential equation consistent with a three-compartment model. The initial [^{3}H]biotin disposition was dominated by a rapidly exchanging pool. Next [^{3}H]biotin disposition was dominated by a more slowly exchanging pool. Finally, [^{3}H]biotin disposition occurred from a third "deep" compartment. The disposition half-lives of [^{3}H]biotin were 0.2 ± 0.02 hours, 1.2 ± 0.1 hours, and 21.9 ± 13.6 hours for the three phases.

The release of [^{3}H]biotin into the medium was temperature dependent (*Figure 2*). When PMBCs were loaded with [^{3}H]biotin at 37°C and then transferred into [^{3}H]biotin-free medium at 4°C, [^{3}H]biotin release from PMBCs was small compared with release at 37°C. After 20 hours at 4°C, 85 ± 7.6% of the intracellular [^{3}H]biotin remained in the cells ($P < 0.01$ compared to controls at 37°C).

2-Deoxy-D-glucose and sodium fluoride inhibit glycolysis and reduce cellular adenosine 5′ triphosphate (ATP) levels.[7,8] In this study, 2-deoxy-D-glucose and sodium fluoride slightly increased (rather than decreased) the rate of [^{3}H]biotin efflux from PMBCs (*Figure 2*). After 20 hours at

37°C in [^{3}H]biotin-free medium containing 2-deoxy-D-glucose and sodium fluoride, only 13 ± 5.1% of the [^{3}H]biotin remained in the cells ($P < 0.05$ compared with controls at 37°C). Incubation with 2-deoxy-D-glucose and sodium fluoride for 20 hours decreased PMBCs viability from 99 ± 0.5% to 74 ± 6.2%. Efflux data were not adjusted for cell viability.

Differences among the three groups (efflux measured at 37°C or 4°C or in the presence of 2-deoxy-D-glucose/sodium fluoride) in intracellular [^{3}H]biotin during the loading phase are not a likely source of the differences observed in the [^{3}H]biotin remaining in the cells of different groups after 20 hours in biotin-free medium. The mass of [^{3}H]biotin in PMBCs during the 2-hour loading was not significantly different among the three groups (*Figure 2*). We estimated the [^{3}H]biotin mass by calculating the areas under the curves of [^{3}H]biotin in PMBCs; the areas under the curves span the range of 1.5 ± 0.3 to 1.7 ± 0.4 (fmol · h)/10^6 cells in the three groups ($P > 0.05$). In these experiments, PMBCs from the same five subjects were used for each of the three treatment groups. PMBCs from individual subjects were not pooled but were analyzed individually. PMBCs were loaded with [^{3}H]biotin under equal conditions (temperature, biotin concentration). The temperature during the loading phase was 37°C for all three treatment groups; of course, the temperature was altered for the 4°C treatment group during the efflux phase.

The efflux of [^{3}H]biotin was not sensitive to either 200 μmol/L phloretin, 200 μmol/L DITS, or 1 mmol/L iodoacetate (data not shown). Incubation with phloretin and DITS for 20 hours decreased PMBCs viability from 99.6 ± 0.3% to 70.1 ± 17.6% and from 99.3 ± 0.7% to 61.3 ± 38.4%, respectively. Efflux data were not adjusted for cell viability.

We sought to determine whether [^{3}H]biotin efflux from PMBCs is enhanced when unlabeled biotin or biotin analogs are added to the extracellular medium at concentrations that saturate the transporter for biotin uptake into PMBCs.[2] When [^{3}H]biotin-loaded PMBCs were incubated in release medium containing unlabeled biotin or biotin analogs at 1 mmol/L, [^{3}H]biotin efflux was significantly more rapid than in control incubations (*Figure 3*). In the presence of biotin analogs, 27 ± 5% to 36 ± 3% of the [^{3}H]biotin remained in the cells after a 30-minute incubation; in controls, 71 ± 4% of the [^{3}H]biotin remained in the cells. These data are consistent with the hypothesis that [^{3}H]biotin efflux is mediated by a transporter that moves across the membrane more rapidly if biotin compounds in the extracellular medium bind to the transporter.

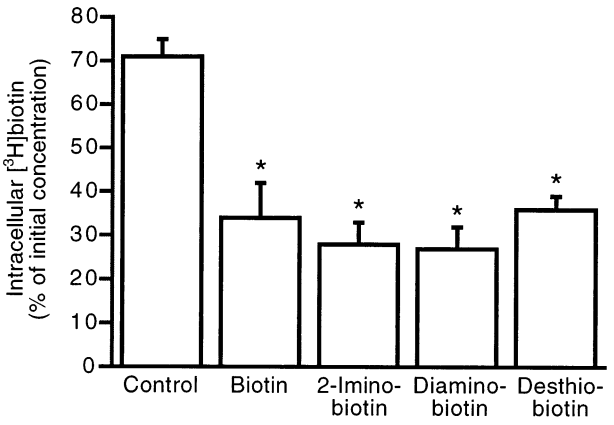

Figure 3 The effect of unlabeled biotin and biotin analogs in the medium on the efflux of [^{3}H]biotin from peripheral blood mononuclear cells (PMBCs). PMBCs were incubated in phosphate buffered saline (PBS) containing 2.75% bovine serum albumin, 5.5 mmol/L D-glucose, and 475 pmol/L [^{3}H]biotin at 37°C for 2 hours. Then the cells were harvested by centrifugation. [^{3}H]Biotin-loaded PMBCs were incubated at 37°C for 0.5 hours in [^{3}H]biotin-free medium containing 2.75% bovine serum albumin and 5.5 mmol/L D-glucose in PBS and 1 mmol/L of either unlabeled biotin, 2-iminobiotin, diaminobiotin, or D,L-desthiobiotin; controls were incubated in biotin-free medium. [^{3}H]Biotin concentrations were measured in the washed cell pellets (values are means ± SD; six aliquots from one set of PMBCs were analyzed). Values are expressed as percentage of the [^{3}H]biotin concentration after 2 hours of loading. *$P < 0.01$ versus control.

Discussion

To our knowledge this is the first published study of biotin efflux from PMBCs. The data from this study provide evidence that biotin efflux from PMBCs is a rapid process mediated by a transmembrane transporter.

Using pharmacokinetic methods, three [^{3}H]biotin compartments can be distinguished in PMBCs. The [^{3}H]biotin compartments may be due to biotin distribution among cellular organelles or to biotin binding to macromolecules. The pharmacokinetic approach used here did not allow specific assignment of the physiologic or anatomic compartments; this limitation is inherent in the technique.[5] We speculate that the terminal half-life of [^{3}H]biotin efflux (γ-phase) is determined by the breakdown of biotin-dependent carboxylases to release free [^{3}H]biotin and the subsequent efflux of [^{3}H]biotin from PMBCs. The half-life of [^{3}H]biotin during the γ-phase of efflux (22 hours) as measured in the present study resembles the half-lives that have been measured previously for pyruvate carboxylase (28 hours) and acetyl-CoA carboxylase (4.6 days).[9–13]

We propose that [^{3}H]biotin is released from PMBCs into the medium by a transporter-mediated process (rather than by free diffusion) on the basis of the decreased rate of [^{3}H]biotin efflux at 4°C compared with 37°C. In addition, our data provide evidence that the transporter for biotin efflux does not require metabolic energy (ATP) directly. When glucose utilization in PMBCs was impaired by 2-deoxy-D-glucose and sodium fluoride, [^{3}H]biotin efflux was even more rapid than in controls ($P < 0.05$). We speculate that the unexpected increased rate of [^{3}H]biotin efflux might be due to the 25% decrease of PMBC viability caused by 2-deoxy-D-glucose and sodium fluoride. However, one cannot rule out the possibility that reduced glucose utilization leads to reduced transformation of biotin to biotinyl-AMP and biotinyl-CoA in PMBCs. Biotinyl-AMP and biotinyl-CoA are substrates for the incorporation of biotin into biotin-dependent enzymes and are putative substrates for the transport of biotin across mitochondria membranes.[1] Hence, if glucose utilization is reduced, biotin-binding to enzymes and biotin transport into mitochondria may be reduced. This may lead to a greater concentration of free biotin in the cell cytosol and a faster efflux into the medium.

Previous studies in various cell types have shown that phloretin inhibits the carrier-mediated uptake of *N*-arachidonoylethanolamine,[14] the uptake of fatty acids,[15–18] the efflux of oleic acid,[19] the efflux of galactose and glucose,[20,21] and anion exchange across membranes.[21] DITS inhibits the carrier-mediated uptake of fatty acids,[22] 2-oxoisocaproate,[23] and biotin.[24] We examined the effects of phloretin and DITS on [^{3}H]biotin efflux from PMBCs. [^{3}H]Biotin efflux from PMBCs was not sensitive to phloretin or DITS in the present study. The results of these efflux studies contrast with previous biotin influx studies.[24] We speculate that DITS and, potentially, phloretin inhibit selectively the cellular influx of biotin. In analogy, *N*-arachidonoylethanolamine enters and leaves cerebellar granule cells using the same transporter.[14] However, only the uptake is sensitive to phloretin.

Conclusion

Our data are consistent with the hypothesis that biotin uptake into PMBCs and biotin efflux from PMBCs are mediated by the same transporter. We found that addition of unlabeled biotin or biotin analogs to the external medium increased the rate of [^{3}H]biotin efflux from [^{3}H]biotin-loaded PMBCs. Analogous interpretations of such "countertransport" effects have been made for the transport of choline in placental brush-border membrane vesicles[25] and in renal brush-border membrane vesicles.[26] The rate of [^{3}H]choline efflux from vesicles was increased if unlabeled choline was added to the extravesicular medium.

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