Impaired Biotinidase Activity Disrupts Holocarboxylase Synthetase Expression in Late Onset Multiple Carboxylase Deficiency*

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Biotinidase catalyzes the hydrolysis of the vitamin biotin from proteolytically degraded biotin-dependent carboxylases. This key reaction makes the biotin available for reutilization in the biotinylation of newly synthesized apocarboxylases. This latter reaction is catalyzed by holocarboxylase synthetase (HCS) via synthesis of 5′-biotinyl-AMP (B-AMP) from biotin and ATP, followed by transfer of the biotin to a specific lysine residue of the apocarboxylase substrate. In addition to carboxylase activation, B-AMP is also a key regulatory molecule in the transcription of genes encoding apocarboxylases and HCS itself. In humans, genetic deficiency of HCS or biotinidase results in the life-threatening disorder biotin-responsive multiple carboxylase deficiency, characterized by a reduction in the activities of all biotin-dependent carboxylases. Although the clinical manifestations of both disorders are similar, they differ in some unique neurological characteristics whose origin is not fully understood. In this study, we show that biotinidase deficiency not only reduces net carboxylase biotinylation, but it also impairs the expression of carboxylases and HCS by interfering with the B-AMP-dependent mechanism of transcription control. We propose that biotinidase-deficient patients may develop a secondary HCS deficiency disrupting the altruistic tissue-specific biotin allocation mechanism that protects brain metabolism during biotin starvation.

In humans, the vitamin biotin is an essential micronutrient that has two different functions in the cell (1). First, it is the cofactor of five biotin-dependent carboxylases: pyruvate carboxylase (PC),3 propionyl-CoA carboxylase (PCC), methylcrotonyl-CoA carboxylase (MCC), and two forms of acetyl-CoA carboxylase (ACC-1 and ACC-2) (2). These enzymes catalyze key reactions in gluconeogenesis, branched chain amino acid catabolism, and fatty acid synthesis and underscore the importance of biotin to metabolic homeostasis (2). Second, biotin is a regulator of the expression of several hepatic proteins that include glucokinase, phosphoenol pyruvate carboxykinase, and most of the proteins involved in biotin metabolism (1, 3–13).

Because of the importance of biotin in cell metabolism, higher organisms face a constant threat to their survival because they are incapable of synthesizing the vitamin. The situation is further complicated by the limited availability of biotin in nature, most of which is protein-bound and not directly accessible (2, 14). During evolution, mammals developed what is known as the biotin cycle, which allows them to cope with the low availability of this critical nutrient (Fig. 1). This system depends on two enzymes: holocarboxylase synthetase (HCS) and biotinidase (1, 2). HCS is responsible for the activation, via biotinylation, of all biotin-dependent carboxylases in human cells. The process takes place in a two-step, ATP-dependent reaction in which biotin is first activated to 5′-biotinyl-AMP (B-AMP) and then transferred to a specific and highly conserved lysine residue in all biotin-dependent carboxylases (2, 15, 16). Biotinidase catalyzes the release of biotin from biotinylated peptides or biocytin (biotinyl-lysine), products generated by intestinal digestion of nutrient proteins or during carboxylase turnover (endogenous biotin recycling) (2, 17).

We recently showed that HCS is an obligate participant in biotin-mediated transcriptional regulation (Fig. 1). The underlying mechanism requires B-AMP, the product of the HCS reaction, which activates a signal transduction cascade involving soluble guanylate cyclase (sGC) and cGMP-dependent protein kinase (PKG) (4, 7). In the presence of biotin, the HCS-sGC-PKG pathway induces the expression of the components of the biotin cycle required for its transport and utilization: the sodium-dependent multivitamin transporter, PC, PCC, ACC, and HCS (3, 5, 13). Paradoxically, biotin deficiency results

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3 The abbreviations used are: PC, pyruvate carboxylase; HCS, holocarboxylase synthetase; MCD, multiple carboxylase deficiency; B-AMP, 5′-biotinyl-AMP; MCC, methylcrotonyl-CoA carboxylase; ACC, acetyl-CoA carboxylase; MCD, multiple carboxylase deficiency; ODQ, 1-H(1,2,4)-oxadiazolo-[4,3-a]quin-axolin-1-one; 8-Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; PVDF, polyvinylidene difluoride.

AMP, PCC, propionyl-CoA-carboxylase; ACC, acetyl-CoA carboxylase; MCC, methylcrotonyl-CoA carboxylase; sGC, soluble guanylate cyclase; PKG, cGMP-dependent protein kinase; ODQ, 1-H(1,2,4)-oxadiazolo-[4,3-a]quin-axolin-1-one; 8-Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; PVDF, polyvinylidene difluoride.
Deficiency of Biotinidase Activity Disrupts HCS Expression

In humans, the biotin cycle can be disrupted by genetic deficiency of holocarboxylase synthetase (HCS deficiency (MIM 253270)) or biotinidase (BTD deficiency (MIM 253260)), resulting in neonatal or juvenile onset forms, respectively, of the disease multiple carboxylase deficiency (MCD) (2, 20, 21). Although the two diseases differ in the age of onset of symptoms, they share a number of clinical and biochemical manifestations, including decreased activities of all carboxylases, organic acidemia, hyperammonemia, dermatitis, alopecia, seizures, and coma. In biotinidase-deficient patients, neurological damage may also include mental retardation, hearing loss, and optic nerve atrophy (2). Although potentially lethal, most of clinical and biochemical manifestations of neonatal and juvenile MCD can be successfully treated with pharmacological doses of biotin.

The biotin-responsiveness of neonatal MCD patients is associated primarily with having at least one allele expressing a mutant HCS with an elevated $K_m$ for biotin, which allows for increased functional activity at high concentrations of biotin (1–5). However, based on the participation of HCS in the biotin-dependent transcriptional regulation of the biotin cycle, we have suggested that the clinical and biochemical deficits in biotin-deficient patients reflect the combined effects of the low affinity of the mutant enzyme for biotin and the concomitant reduction in carboxylase and HCS mRNA levels (3, 4). In biotinidase-deficient patients, the biotin cycle is largely intact because free biotin can be successfully utilized for the biotinylation of carboxylases. Here the deficit has been thought to be in the inadequacy of the biotin supply because of the inability to recycle protein-bound biotin from endogenous or nutrient sources (2). Therefore, supplementation with biotin at pharmacologic doses is thought to compensate for the loss of access to the additional biotin that would normally be available from protein sources (2). Although the ultimate consequence of biotinidase deficiency is the interruption of the metabolic pathways where biotin-dependent carboxylases participate, the clinical manifestations that distinguish this disorder from HCS deficiency, especially in relation to neurological presentation, are not fully understood (22).

In this work, we use fibroblasts from a biotinidase-deficient patient as an experimental model to study the role of this enzyme in carboxylase biotinylation and in HCS-sGC-PKG-dependent expression of biotin-dependent carboxylases and HCS under conditions of biotin deficiency and supplementation. Our results show that in biotinidase-deficient cells, biotin starvation results in a more rapid reduction in carboxylase biotinylation and in the expression of PC and MCC than in normal fibroblasts. We also demonstrate that in biotinidase-deficient cells the expression and activity of HCS is lower than in control cells. We propose that, in the absence of biotin supplementation, biotinidase-deficient patients may develop a secondary HCS deficiency that, combined with the primary biotinidase deficiency, may disrupt the altruistic regulation of biotin utilization that protects brain metabolism against vitamin starvation.

EXPERIMENTAL PROCEDURES

Materials—Biotin, biocytin, 1-H(1,2,4)-oxadiazolo-[4,3-a]quinazolin-1-one (ODQ), and 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP) were from Sigma-Aldrich. d-[8,9-3H]biotin (34.0 Ci/mmol) was purchased to Amersham Biosciences. The biotinidase-deficient cell line MCD-BD was from the Montreal Children’s Hospital Cell Repository for Mutant Human Cell Strains, and the HCS-deficient cell line MCD-MK (23) and the rabbit antibody to HCS (24) were received previously. Human normal fibroblasts were obtained from G. Soldevila (Universidad Nacional Autónoma de México), Escherichia coli C-124 cells have been previously described (16, 25). Cell cultures were maintained in α-minimum Eagle’s...
medium containing high glucose (Invitrogen; biotin concentra-
tion, 0.40 μM) supplemented with 10% heat-inactivated fetal
bovine serum, 100 units/ml penicillin, and 100 μg/ml strepto-
mycin (biotin-replete medium). Biotin-deficient medium was
prepared using biotin-free minimum Eagle’s medium, dialyzed
fetal bovine serum (Invitrogen), and the same antibiotic
concentration.

Cell Culture Experiments—The methods for biotin starva-
tion of cell cultures are essentially as described previously (3, 4).
Briefly, the cells were grown in biotin-replete or biotin-defi-
cient medium at 37 °C with 5% CO₂ for up to 13 days. The
medium was changed at 3-day intervals. For carboxylase bioti-
nylation recovery experiments, the cells grown in biotin-defi-
cient medium were stimulated with biotin or biocytin, at con-
centrations from 1 to 100 nM for 24 h.

Western Blot Analysis for HCS Expression and Carboxylase
Biotin Content—Crude extracts from human cell cultures (30 –
100 μg of total protein) were fractionated by SDS-polyacryl-
amide gel electrophoresis and transferred to a polyvinylidene
difluoride (PVDF) membrane (Millipore). The membranes
were incubated in a 1:3000 solution of rabbit HCS antibody or
with a 1:2000 solution of streptavidin-AP conjugate (Roche
Applied Science) or with a 1:500 solution of goat MCC or PCC
antibodies (Santa Cruz Biotechnology). Visualization of HCS
bands was performed using a BM chemiluminescence Western
blotting kit (Roche Applied Science). Biotin-containing bands
were quantitated using an FX image analyzer (Bio-Rad) as
described above. The protein concentration used in these
experiments was determined using a ND-1000 spectrophotom-
eter (Nanodrop Technologies, Inc.), and confirmation of equal
amounts of total protein in every lane was done by staining the
gels with Coomassie Blue before protein transfer to PVDF
membranes.

Effect of Biotin and cGMP on Carboxylase Expression in Bio-
tin-starved Cells—To determine the involvement of sGC on
the recovery of biotinyltion and carboxylase protein levels, biotin-
starved normal fibroblasts were treated with 50 μM ODQ, a
specific inhibitor of sGC, for 3 h (4). After this period, 1 μM
biotin was added to the medium for 48 or 72 h, and the effect on
MCC protein levels was compared with biotin-deficient cells
stimulated by biotin without ODQ and cells grown continu-
ously in normal medium (control cells). Alternatively,
MCD-BD cells grown in biotin-free medium for 13 days were
stimulated with 1 μM biotin or 1 mM B-Br-cGMP, a nonhydro-
lyzable analogue of cGMP. The cells were harvested after 48 or
72 h, and the MCC protein levels were determined as described
above.

Reverse Transcription-PCR—Procedures for RNA isolation,
cDNA synthesis, and PCR have been previously described (3, 4).
5 μg of total RNA and 0.5 μM gene-specific oligonucleotide
primers were used for cDNA synthesis and 0.3 μM specific
sense and antisense primers were used to give 200–300 bp of
PCR products. The oligonucleotides used to amplify human
mRNA were: HCS: 5’-CCC GAG CAT CGT CTC CGT GAT
GGG-3’ and 5’-CCC AAC CCT TTT ACC GCC GTT TGG
GGA-3’ (Tₘ = 58 °C); Biotinidase, 5’-ATC TAT GAA CAG
CAA GTG ATG ACT-3’ (Tₘ = 66 °C) and 5’-AGG GAC CAG
GTT GAA ATG GTC ATA-3’ (Tₘ = 70 °C); β-actin: 5’-GGG
TCA GAA TTC CTA TG-3’ and 5’-GGT CTC AAA CAT GAT
CTG GG-3’ (Tₘ = 58 °C). PCR products were separated on 1%
agarose gels and stained with ethidium bromide. The amount of
PCR product was determined by densitometry by using a Fluor-
S-imager (Bio-Rad) as previously described (3, 4). The proce-
dure was validated in prior studies by PCR amplification of
different concentrations of cDNA fragments of HCS, biotini-
dase, and β-actin (data not shown). The number of PCR cycles
was also varied and plotted against fluorescence intensity to
ensure that experiments were done within the exponential
phase. For every experiment, the constitutive β-actin mRNA
was used as the reference cellular transcript. It was present at
equivalent levels in all RNA samples.

HCS Activity Assay Using p67 as Biotinyltion Substrate—To
determine HCS activity in normal and MCD-BD fibroblasts, we
used a modification of the protocol described previously (16,
25). Briefly, a pFLAG vector (Sigma) containing a cDNA frag-
ment encoding the last 67 amino acids (640–703) of the
α sub-unit of human PCC was used to transform wild type E. coli XL1
Blue and E. coli C-124, a mutant strain unable to synthesize
dethiobiotin, an essential intermediate in the production of
biotin. Log phase XL-1 and C-124 cultures in L-broth medium
were transferred to a biotin-free medium (7.5 mM (NH₄)₂SO₄,
33 mM KH₂PO₄, 60 mM K₂HPO₄, 1.7 mM sodium citrate, 1 mM
MgSO₄, 0.2% dextrose, 0.1% casamino acids) and 2 mM isopro-
pyl β-D-thiogalactopyranoside and incubated at 37 °C for 4 h.
The cells were sonicated three times with 10-s pulses and cen-
trifuged at 15,000 rpm for 20 min. The proteins in the superna-
tant were resolved by 12% acrylamide gel electrophoresis (100
μg of total protein/lane) and transferred to a PVDF membrane.
Two biotinylated proteins are possible in cells expressing p67:
p67, at 14 kDa, and BCCP, the 18-kDa subunit of E. coli ACC.
To detect their positions on the gel, one lane containing pro-
teins expressed by XL1 cells transformed with p67 was cut off
from the membrane and incubated with streptavidin-AP to
detect the biotinylated proteins (see Fig. 6A). The lower band,
corresponding to p67, was used as a reference to cut out the
section of the membrane in adjacent lanes containing unbio-
tinylated or apo-p67 expressed by E. coli C-124. The membrane
pieces containing apo-p67 were used, in solid phase format, for
HCS assays. HCS activity was monitored by incubating the
membrane pieces for 1 h at 25 °C in 150 μl of reaction buffer
containing Tri-HCl, pH 8.0, 50 mM reduced glutathione, 22.5
mM MgCl₂, 5 mM ATP, 1–3 μCi of [³H]biotin, and 100 μg of
total protein of crude extracts from normal or MCD-BD fibro-
blasts. For these experiments cells grown in biotin-supple-
mented medium were preincubated for 6 h with 1 μM nonra-
dioactive biotin and 63 μM cycloheximide to block free
biotinylation sites and prevent de novo carboxylases synthesis
(5). Crude extracts were prepared as previously described and
passed twice through an Amicon ultra centrifugal device (Mil-
lipore) to eliminate nonradioactive biotin. The radioactive bio-
tin incorporated into the membrane-bound p67 was estimated
using a Beckman LS 6500 scintillation counter. Under these
conditions, the p67 was in excess, and the assay was linear for
the 1 h of incubation.

cDNA and Genomic DNA Sequencing—To determine the
mutations responsible for the phenotype of MCD-BD cells, the

Deficiency of Biotinidase Activity Disrupts HCS Expression
Deficiency of Biotinidase Activity Disrupts HCS Expression

Molecular and Functional Characterization of Cell Line MCD-BD

A, partial biotinidase amino acid sequence (residues 140–180 and 420–460) localizing substitutions A171T and D444H found in the cell line MCD-BD. The corresponding nucleotide sequence is shown above the amino acid sequences with mutations indicated in bold characters. B, representative Western blot experiment showing the effect of biotinidase deficiency (MCD-BD cells) in the biotinylation of PC, MCC, and PCC following growth in biotin replete (0.40 μM) medium. The MCD-MK cells are from a patient with HCS deficiency (3). The changes in streptavidin-AP-reactive MCC/PCC bands were analyzed by densitometry. The values below the lanes correspond to the relative densitometry value (RDV) normalized to the value observed in normal fibroblasts. Each lane contains 50 μg of crude cell lysate.

The biotinidase cDNA was cloned in the pGEM vector (Promega). Biotinidase exons were amplified from genomic DNA as previously described (26, 27) and subcloned also in pGEM. Both cDNA and exons were sequenced at Laragen (Los Angeles, CA).

**Statistical Analysis**—All of the experiments were done in triplicate and at least three different times. The results of biotin starvation on mRNA were normalized to β-actin mRNA and expressed as a percentage of mRNA levels observed in cells grown in biotin-replete medium. The data are presented as the mean of three different experiments ± S.E. unless otherwise indicated. Statistical significance of p67 biotinylation results obtained with normal or MCD-BD cells were analyzed at 0.05 and 0.01 levels of significance using Student’s t test one-way ANOVA.

**RESULTS**

**Molecular and Functional Characterization of the Biotinidase-deficient Cell Line MCD-BD**—To characterize the cell line MCD-BD used as an experimental model in this study, we first identified the mutations responsible for biotinidase deficiency by sequencing the cDNA encoding this enzyme. This procedure resulted in the identification of a transversion 1330G→A, resulting in a substitution of His for Asp444 (D444H), and a single base transition 511G→A, resulting in a substitution of Thr for Ala171 (A171T) (Fig. 2A). These mutations have been previously reported (26) and are considered a common cause of profound biotinidase deficiency in children ascertained by newborn screening in the United States (28). Because these mutations were originally described as a double mutation allele (28), we sequenced all four biotinidase exons from genomic DNA to search for another mutation. This procedure confirmed the identified mutations and did not reveal the presence of any other mutation.

To verify the impact of these mutations, we tested the biotinylation status of the carboxylases PC, PCC, and MCC in MCD-BD cells and compared the results with normal fibroblasts (positive control) and MCD-MK fibroblasts (negative control). The latter cells have unbiotinylated carboxylases in the standard biotin-replete medium caused by a homozygous, high Km, R508W mutation in HCS but are restored to normal in medium containing 100× biotin (23). The cells were grown in biotin-replete medium to 80% confluence, and total proteins from cell extracts were resolved by polyacrylamide gel electrophoresis. The biotin content in carboxylases was visualized by Western blot using streptavidin-AP. Three bands were identified in normal cells corresponding to PC (128 kDa), MCC-α subunit (76 kDa), and PCC-α subunit (72 kDa). As we previously reported, ACC (265 kDa) is not detected using this colorimetric assay (3). RCC and PCC appear as a broad band or doublet, clearly distinguishable from the fainter PC band (Fig. 2B). In contrast, MCD-BD cells exhibited reduced MCC-PCC biotinylation in biotin-replete medium, to approximately two-thirds of the levels observed in the normal fibroblasts. As expected, MCD-MK cells showed almost complete absence of biotin incorporation into PC, MCC and PCC (Fig. 2B). These results suggest that profound biotinidase deficiency results in a reduced level of carboxylase biotinylation in human fibroblasts in culture, even when grown in the presence of excess biotin.

**Biotinidase Deficiency Hinders Recovery of Carboxylase Biotinylation in Biotin-deficient Cells in Response to Biotin or Biotin Stimulation**—The above experiment demonstrated a biotinylation defect in the MCD-BD cells that extends beyond the immediacy of the defect in releasing protein-bound biotin. To determine whether this is the result of a deficient recycling of biotin, we examined the ability of biotin-starved cells to recover carboxylase biotinylation after stimulation with biotin or biocytin. Previous studies by us and others established that nutrient biotin deficiency results in a reduction in biotinylated PC, PCC, and MCC (3, 29, 30). In this study, we have focused on the combined PCC-MCC biotinylation to monitor biotin status in our experiments. The cells were grown in biotin-deficient medium for 13 days and then stimulated with biotin or biocytin for 24 h at concentrations ranging from physiological to pharmacological (1, 10, and 100 nm). Total protein extracts from the cell cultures were separated by gel electrophoresis, and the recovery in PCC-MCC biotinylation was determined with streptavidin-AP. In these experiments, the results are normalized with respect to the values obtained by normal fibroblasts in normal medium. Normal fibroblasts incubated in biotin-deficient medium showed PCC-MCC biotinylation of less than 25% of the level observed in control cells. When the biotin-starved cells were stimulated with 1, 10, and 100 nm biotin, the biotin content in PCC-MCC increased to 26 ± 2, 87 ± 14, and 103 ± 11% of control values, respectively (Fig. 3A). Similar results were obtained when biocytin, instead of biotin, was used as the
Deficiency of Biotinidase Activity Disrupts HCS Expression

supplement at the same concentrations, with the cells reaching 24 ± 3, 79 ± 15, and 80 ± 13% of control values, respectively (Fig. 3B). These results confirm that normal cells are equally efficient in promoting carboxylase biotinylation using free biotin or biocytin as the biotin source. Next, we determined the recovery of carboxylase biotinylation in MCD-BD cells under the same experimental conditions. Biotin starvation reduced carboxylase biotin content to 37 ± 5% of that observed in biotin-replete medium. Strikingly, biotinidase-deficient cells showed a poor response to biotin. When the biotin-starved cultures were stimulated with 1, 10, and 100 nM biotin, PCC-MCC biotinylation values were 33 ± 6, 45 ± 10, and 48 ± 2%, respectively (Fig. 3C). When 1, 10, and 100 nM biocytin was added to the medium, carboxylase biotinylation was not significantly affected (27 ± 7, 37 ± 9, and 38 ± 16%) (Fig. 3D). These results are consistent with the block in biotinidase, resulting in the inability to release biotin from biocytin for use in the biotinylation of the carboxylases. However, the reduced biotinylation in biotin-replete medium and the failure to readily respond to free biotin following biotin starvation implicates a more complex process.

Effect of Biotin Deficiency and Supplementation on Carboxylase Protein Levels in Normal and Biotinidase-deficient Cells—We explored the relationship between the lack of response to biotin stimulation observed in MCD-BD cells and the size of the pool of apocarboxylases available for biotinylation. In this experiment we studied the effect of biotin on the recovery of carboxylase biotinylation and PC and MCC protein levels in biotin-starved normal and MCD-BD cells. As described above, Western blot analysis using streptavidin-AP revealed that biotin deficiency reduced the level of biotinylated PC, MCC and PCC in normal cells. The addition of 1 μM biotin (concentra-
Deficiency of Biotinidase Activity Disrupts HCS Expression

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**Effect of Biotin Deficiency on HCS and Biotinidase mRNA Levels in Normal and Biotinidase-deficient Fibroblasts**—To test this hypothesis, we examined the impact of biotin deficiency on the level of HCS mRNA to determine whether the biotin-dependent transcriptional regulation of the biotin cycle is impaired in these cells. We used HCS mRNA as the indicator for this experiment because HCS is the key regulatory enzyme in biotin-dependent transcription and is the first enzyme of the biotin cycle subject to transcriptional control via the HCS-sGC-PKG pathway. First, we examined the time course of the effect of biotin starvation on the level of HCS mRNA, as well as biotinidase and β-actin mRNA, in normal cells. This was done by growing the cells in biotin-deficient medium for 13 days and assessing the mRNA levels at intervals during the course of the experiment, as described under “Experimental Procedures.” The results were normalized with respect to initial mRNA levels for each culture to facilitate comparison of the results obtained with different cultures. In normal cells, biotin starvation reduced the level of HCS mRNA in a gradual manner, with 95% of the starting level remaining at day 3 and leveling off at 55–60% by days 11–15 (Fig. 5A). No significant change was observed in biotinidase or β-actin mRNA levels during the course of the experiment. In MCD-BD cells, the level of HCS mRNA in biotin-replete medium was 52 ± 3% lower than in normal fibroblasts (data not shown). Normalized to the starting level, HCS mRNA levels also fell more rapidly following the shift to biotin-deficient medium, showing a detectable reduction in the first day of the experiment and reaching 60% of the starting level by day 3 and 20% by day 15 (Fig. 5B). These results suggest that HCS mRNA levels are more easily reduced by biotin starvation in biotinidase-deficient cells than in normal fibroblasts and revealed the unexpected result that HCS mRNA was reduced even in high biotin medium. Biotinidase and β-actin mRNA levels, as observed for normal fibroblasts, were not affected by biotin deficiency, consistent with our previous finding that biotinidase expression is not regulated by biotin availability and the HCS-sGC-PKG pathway (3).

**HCS Protein Levels and Biotinylat**

**ion Activity in Biotinidase-deficient Cells**—The rapid reduction in HCS mRNA in MCD-BD cells during biotin starvation provides an explanation for the reduced level of biotinylated carboxylases in these cells compared with normal cells, because B-AMP produced by HCS is required to maintain the HCS-sGC-PKG-dependent expression of carboxylases (3, 4). We therefore determined whether the reduction in HCS mRNA translated into a lower level of enzyme function. To determine HCS activity, we made use of p67, the carboxyl-terminal 67-amino acid fragment of human α subunit of PCC, as the substrate for biotinylation (16, 25) (Fig. 6A). Total cell extracts prepared from normal or MCD-BD fibroblasts were incubated in the presence of p67 bound to PVDF membrane and 1 μCi of [3H]biotin and ATP, as described under “Experimental Procedures.” The amount of membrane-fixed radioactivity, corresponding to the amount of [3H]biotin incorporated in p67, was taken as a measure of HCS activity present in the different cell extracts. In this experiment, extracts from biotin-replete cells were used to minimize the presence of apocarboxylases in the samples, which would otherwise compete for substrate (5, 29). Extracts from normal cells catalyzed the incorporation of 5000 ± 500 cpm of [3H]biotin in p67, whereas MCD-BD cell extracts showed 50% reduction in biotinylatation activity, incorporating 2320 ± 300 cpm into the p67 substrate (Fig. 6B). By Western blot analysis using HCS antibody, we confirmed that the reduction in biotinylation activity is associated with a reduction in the amount of HCS protein. Biotinidase-deficient cells showed one-third of the HCS immunoreactive material expressed by normal cells (Fig. 6C).

**DISCUSSION**

In this work, we investigated the impact of biotinidase deficiency on the maintenance of biotinylatation and expression of carboxylases and regulation of the biotin cycle in human cells under conditions of biotin starvation and replenishment. Our results show that biotin deficiency, as expected, reduces carboxylase biotinylatation in both normal and biotinidase-deficient cells. However, whereas in normal fibroblasts this effect is efficiently reversed after incubation with physiological concentrations of biotin or biocytin, in biotinidase-deficient cells it is not. These cells are unable to recover carboxylase biotinylation levels with biocytin and showed only partial recovery after a prolonged incubation with pharmacological doses of free biotin. The lack of response to biocytin is understood because biotinidase is required to release biotin from this compound, but the reduced response to free biotin demonstrates a more complex impact of biotinidase deficiency than is anticipated from a simple functional defect in the enzyme. Here we showed that MCD-BD behavior is associated with dimi-
Deficiency of Biotinidase Activity Disrupts HCS Expression

FIGURE 6. Holocarboxylase synthetase activity and protein in MCD-BD cells. HCS activity was determined in a solid phase assay in which p67 bound to PVDF membrane was used as substrate for biotinylation. A, preparation of p67 substrate. A PVDF membrane was prepared by transfer from a polyacrylamide gel following electrophoresis of extracts of E. coli XL-1 (wild type) or C-124 cells (biotin auxotroph) expressing p67. The two protein bands correspond to BCCP (upper band, 18 kDa) and p67 (lower band, 14 kDa). The membrane was developed with streptavidin-AP (left two lanes) to detect biotinylated proteins or anti-FLAG antibody (right lane) to detect unbiotinylated p67. In XL-1 cells both bands are biotinylated because of endogenously synthesized biotin. In C-124 cells, in which p67 expression was induced in biotin-free medium, only the upper band is biotinylated. The lane on the right confirms the presence of unbiotinylated p67 in the C-124 cells using the FLAG antibody. B, HCS activity of fibroblast cultures. PVDF membrane-bound p67 was incubated in the presence of \([3H] biotin, ATP, and total protein extracts from human fibroblasts. The amount of \([3H] biotin incorporated in p67 by normal fibroblasts (white bar) or MCD-BD cells (black bar) was determined using a liquid scintillation counter. Differences between normal and MCD-BD cells shown to be statistically significant (p < 0.05). C, detection of HCS protein in fibroblast cultures. The level of HCS protein in normal fibroblasts and MCD-BD cells was visualized by Western blot using an antibody against the amino-terminal region of HCS.

MCD-BD cells were expected to express normal levels of HCS, because this enzyme is not directly affected by biotinidase mutations (31). Instead, we observed a reduced level of HCS mRNA and protein and a reduced level of HCS enzyme activity in MCD-BD cells, even in high biotin medium. Further, MCD-BD cells were found to recover poorly on return to biotin-rich medium, despite a seemingly intact ability to utilize free biotin. We propose that biotinidase deficiency produces a cascade of events that begins with a block in the utilization of protein-bound biotin. The reduced access to this biotin source mimics a partial, intracellular biotin deficiency which in turn reduces the availability of B-AMP required to fuel the HCS-sGC-PKG pathway sufficiently to maintain the optimal expression of HCS and apocarboxylase genes, resulting in continued down-regulation of the pathway. A similar outcome in wild type cells, illustrated by reduction in MCC protein, could be obtained by ODQ inhibition of sGC, and full restoration of MCC protein in MCD-BD cells could be obtained on incubation of cells in 8-Br-cGMP. We surmise that once de novo synthesis of carboxylases is halted, the apparent reduction in carboxylase protein is likely the result of intracellular protein turnover. Because in normal cells, the MCC protein level was not affected by biotin deficiency, we suggest that MCC expression is maintained through endogenous biotin recycling via biotinidase activity. On the other hand, MCD-BD cells, unable to recycle protein-bound biotin, are prone to exhibit this decreased expression of biotin acceptor carboxylases and HCS, the enzyme responsible for biotinylating activity and the key component of the HCS-sGC-PKG signal transduction pathway.

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The outcome of the effect of biotinidase deficiency on the regulation of the HCS-sGC-PKG pathway is reduced expression of HCS, PC, PCC, ACC, and sodium-dependent multivitamin transporter (3–5), producing a net deficiency of biotinylated carboxylases below the threshold required for effective contribution to intermediary metabolism. This disruption in the regulation of the biotin cycle provides an explanation for the compromised clinical state of patients with biotinidase deficiency. Although most dietary biotin is protein-bound (2, 14), a simple supplementation of equivalent amounts of free biotin would have been expected to be sufficient to treat the disease. Instead, as with HCS deficiency, persistent treatment with pharmacologic doses of biotin is required to keep patients free of metabolic symptoms (2). Our studies suggest that the role of biotin supplementation is not simply to provide the product of the defective biotinidase, but it is also to overcome the repression of HCS and apocarboxylase expression that accompanies dietary biotin deficiency or genetic defects of HCS.

We are led to propose, therefore, that the constitutive expression of biotinidase may contribute to cell survival by becoming, during periods of insufficient free biotin, the primary source of biotin to fuel carboxylase biotinylation. Biotinidase is the only enzyme of the biotin cycle that is not under the transcriptional control of the HCS-sGC-PKG pathway and thus endogenous biotin recycling is expected to continue independently of biotin availability in the cell. However, in biotinidase deficiency, the brain remains as susceptible as other tissues to biotin insufficiency, resulting in a constellation of neurological abnormalities, including hearing loss, optic atrophy, loss of vision, basal ganglia calcifications, cerebral edema, cerebral atrophy, and subacute necrotizing encephalopathy (Leigh syndrome) (32, 33), that are not normally observed either in nutri-
ent biotin deficiency or in HCS deficiency. Although biotinidase mRNA and protein are expressed throughout the brain, they are most abundant in centers of the auditory and visual activity, including dorsal and ventral cochlear nuclei, superior olivary complex, and vestibular nucleus (34). Because these areas also seem to contain more biotin than other regions of the brain, it is possible that regulation of the biotin cycle may be of particular relevance for these cells (26, 35). We suggest that biotinidase deficiency in combination with limited amounts of biotin may lead to the development of a secondary HCS deficiency that may compromise the survival of cells with a higher metabolic demand such as neurons. The loss of the privileged status of the brain in biotinidase deficiency is supported by necropsy data, showing an almost nonexistent PCC activity in the brain, whereas in kidney and liver, this enzyme activity is only moderately reduced (29 and 42%, respectively), with respect to the levels observed in control individual (33).

Recently, other biotin-responsive disorders have been described, including biotin-responsive basal ganglia disease and biotin-responsive limb weakness (36–39). These diseases are characterized by subacute encephalopathy, mental retardation, severe cogwheel rigidity, dystonia, and quadripareis. Because symptoms can be prevented or reversed with pharmacological doses of biotin and because some cases of biotinidase deficiency have been associated with progressive encephalopathy (40, 41), it has been suggested that the biotin pathway is involved in these disorders. Yet the relationship to abnormalities in biotinidase, HCS, or even biotin transport has been elusive (36, 37, 42). It is possible that these disorders represent different degrees of the same disease in which the delicate balance between utilization of exogenous vitamin and the recycling of the endogenous vitamin have been disrupted, affecting the expression of HCS and the biotin cycle.

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REFERENCES

1. Pacheco-Alvarez, D., Solórzano-Vargas, R. S., and Leon-Del-Río, A. (2002) Arch. Med. Res. 33, 439–447
2. Wolf, B. (2001) in The Metabolic and Molecular Bases of Inherited Disease (Scriver, C., Beaudet, A. L., William, S., and Valle, D., eds) pp. 3935–3962, McGraw-Hill Medical Publishing Division, New York
3. Pacheco-Alvarez D., Solórzano-Vargas, R. S., Gravel, R. A., Cervantes-Roldán, R., Velázquez, A., and Leon-Del-Río, A. (2004) J. Biol. Chem. 279, 52312–52318
4. Solórzano-Vargas, R. S., Pacheco-Alvarez, D., and Leon-Del-Río, A. (2002) Proc. Natl. Acad. Sci. U. S. A 99, 5325–5330
5. Pacheco-Alvarez, D., Solórzano-Vargas, R. S., González-Noriega, A., Michalak, C., Zempleni, J., and León-Del-Río, A. (2005) Mol. Genet. Metab. 85, 301–307
6. Chauhan, J., and Dakshinamurti, K. (1991) J. Biol. Chem. 266, 10035–10038
7. Collins, J., Piaetta, E., Green, R., Morell, A., and Stockert, R. (1988) J. Biol. Chem. 263, 11280–11283
8. Deodhar, A. D., and Mistry, S. P. (1970) Life Sci. II 9, 581–588
9. Dakshinamurti, K., Tarrant-Litvak, L., and Hong, H. C. (1970) Can. J. Biochem. 48, 493–500
10. Borboni, P., Magnaterra, R., Rabini, R. A., Staffolani, R., Porzio, O., Sesti, G., Fusco, A., Mazzanti, L., Lauro, R., and Marlier, L. N. (1994) Acta Diabetol. 33, 154–158
11. De la Vega, L. A., and Stockert, R. J. (2000) Am. J. Physiol. 279, C2037–C2042
12. Maeda, Y., Kawata, S., Inui, Y., Fukuda, K., Igura, T., and Matsuzyawa, Y. (1996) J. Nutr. 126, 61–66
13. Rodríguez-Melendez, R., Caño, S., Mendez, S. T., and Velazquez, A. (2001) J. Nutr. 131, 1909–1913
14. Pispas, J. (1965) Ann. Exp. Med. Biol. Fenn. 43, 1–39
15. Chapman-Smith, A., and Cronan, J. E., Jr. (1999) Trends Biochem. Sci. 24, 359–363
16. Leon-Del-Río, A., and Gravel, R. A. (1994) J. Biol. Chem. 269, 22964–22968
17. Cole, H., Reynolds, T. R., Lockyer, J. M., Buck, G. A., Denson, T., Spence, J. E., Hymes, J., and Wolf, R. (1994) J. Biol. Chem. 269, 6566–6570
18. Chiang, G., and Mistry, S. (1974) Proc. Soc. Exp. Biol. Med. 146, 21–24
19. Hassel, B. (2000) Mol. Neurobiol. 22, 21–40
20. Wolf, B., Grier, R., Secor McVoy, J. R., and Heard, G. (1985) J. Inherit. Metab. Dis. 8, 53–58
21. Dupuis, L., Campeau, D., Leclerc, D., and Gravel, R. A. (1999) Mol. Genet. Metab. 66, 80–90
22. Sander, J. E., Packman, S., and Townsend, J. J. (1982) Neurology 32, 878–880
23. Dupuis, L., Leon-Del-Río, A., Leclerc, D., Campeau, E., Sweetman, L., Saudubray, J. M., Herman, G., Gibson, K. M., and Gravel, R. A. (1996) Hum. Mol. Genet. 5, 1011–1016
24. Naram, M. A., Dumas, R., Ayer, L. M., and Gravel, R. A. (2004) Hum. Mol. Genet. 13, 15–23
25. Leon-Del-Rin, A., Leclerc, D., Akerman, B., Wakamatsu, N., and Gravel, R. A. (1995) Proc. Natl. Acad. Sci. U. S. A 92, 4626–4630
26. Wolf, B., Jensen, K. P., Barshop, B., Blitzer, M., Carlson, M., Goudie, D. R., Gokcay, G., Hare, M., Millot, M., Akerman, B., Wakamatsu, N., and Gravel, R. A. (1995) J. Biol. Chem. 270, 22964–22968
27. Wolf, B., Grier, R., Secor McVoy, J. R., and Heard, G. (1985) J. Inherit. Metab. Dis. 8, 53–58
28. Norrgard, K. J., Pomponio, R. J., Swango, K. L., Hymes, J., Reynolds, T., Buck, G. A., and Wolf, B. (1998) J. Nutr. Sci. Vitaminol. 44, 326–337
29. Schulz, P. E., Weiner, S. P., Belmont, J. W., and Fishman, M. A. (1988) Neurology 38, 1326–1328
30. Baumgartner, E. R., Suormala, T. M., Wick, H., Probst, A., Blauenstein, U., Bachmann, C., and Vest, M. (1989) Pediatr. Res. 26, 260–266
31. Heller, A. J., Stanley, C., Shaia, W. T., Sismanis, A., Spencer, R. F., and Wolf, B. (2002) Hear. Res. 173, 62–68
32. Bhagavan, H. N., and Coursin, D. B. (1970) J. Neurochem. 17, 289–290
33. Ozand, P. T., Gascon, G. G., Alessa, M., Joshi, S., AlJishi, E., Bakheet, S., Al Watanan, J., Al-Kawi, M. Z., and Dabbagh, O. (1998) Brain 121, 1267–1279
34. Subramanian, V. S., Marchant, J. S., and Said, H. M. (2006) Am. J. Physiol. 291, C581–C589
35. Adhisivam, B., Mahto, D., and Mahadevan, S. (2007) Indian Pediatr. 44, 228–230
36. Straussberg, R., Shorer, Z., Weitz, R., Basel, L., Kornreich, L., Corie, C. I., Harel, L., Djalietdi, R., and Amir, J. (2002) Neurology 59, 983–989
37. Suchy, S. F., McVoy, J. S., and Wolf, B. (1985) Neurology 35, 1510–1511
38. Héron, B., Gautier, A., Dulac, O., and Ponsot, G. (1993) Arch. Fr. Pediatr. 50, 875–878
39. Lo, W., Kadlecek, T., and Packman, S. (1991) J. Nutr. Sci. Vitaminol. (Tokyo) 37, 567–572