Biotin Deficiency Inhibits Heme Synthesis and Impairs Mitochondria in Human Lung Fibroblasts[1]

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Abstract

Four of the 5 biotin-dependent carboxylases (BDC) are in the mitochondria. BDC replace intermediates in the Krebs [tricarboxylic acid (TCA)] cycle that are regularly removed for the synthesis of key metabolites such as heme or amino acids. Heme, unlike amino acids, is not recycled to regenerate these intermediates, is not utilized from the diet, and must be synthesized in situ. We studied whether biotin deficiency (BD) lowers heme synthesis and whether mitochondria would be disrupted. Biotin-deficient medium was prepared by using bovine serum stripped of biotin with charcoal/dextran or avidin. Biotin-deficient primary human lung fibroblasts (IMR90) lost their BDC and senesced before biotin-sufficient cells. BD caused heme deficiency; there was a decrease in heme content and heme synthesis, and biotin-deficient cells selectively lost mitochondrial complex IV, which contains heme-*a*. Loss of complex IV, which is part of the electron transport chain, triggered oxidant release and oxidative damage, hallmarks of heme deficiency. Restoring biotin to the biotin-deficient medium prevented the above changes. Old cells were more susceptible to biotin shortage than young cells. These findings highlight the biochemical connection among biotin, heme, and iron metabolism, and the mitochondria, due to the role of biotin in maintaining the biochemical integrity of the TCA cycle. The findings are discussed in relation to aging and birth defects in humans. J. Nutr. 137: 25–30, 2007.

Introduction

Biotin is one of the least-studied vitamins, particularly in relation to mitochondrial function and the extent of its nutritional deficiency in humans (1). Biotin deficiency (BD)[2] in humans appears more common than previously thought (2), as is marginal deficiency during human pregnancy (2). Marginal BD in rodents is teratogenic even though the dams appear normal (2,3). The high biotin requirement of the developing human fetus may explain the susceptibility of embryonic development to BD (4).

Although the role of biotin in mitochondrial biochemistry is well established (5), few studies of BD have focused on how such a deficiency affects mitochondria (6), oxidative stress, and aging (7). Biotin is a coenzyme in 5 different biotin-dependent carboxylases (BDC), which catalyze carboxylation reactions (5): pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC), 3-methylcrotonyl-CoA carboxylase (MCC), acetyl-CoA carboxylase (ACC)-2, and ACC-1. The first 4 are located in the mitochondria. PC, PCC, and MCC catalyze anaplerotic reactions and replenish tricarboxylic acid (TCA) cycle intermediates (8). The fifth BDC, ACC-1, is located in the cytosol and is important for fatty acid metabolism, as is ACC-2. The carboxyl group of carboxy-biotin is transferred by PC to pyruvate to form oxalacetate; by PCC to propionyl-CoA to form succinyl-CoA; and by MCC to 3-methylcrotonyl-CoA to form 3-methyglutaconyl-CoA, which is metabolized to acetyl-CoA (5). All feed directly into the TCA cycle (5). This study examines the effects of BD on mitochondria and cellular senescence resulting from modulations of anaplerotic carboxylation reactions.

BD has a detrimental effect on the level of TCA cycle intermediates. A deficiency in PC directly decreases production of oxaloacetate. A deficiency in PCC decreases production of succinyl-CoA and causes propionyl-CoA to accumulate, which interacts via a side reaction with oxaloacetate to form methylcitrate. Additionally, low activity of MCC causes methylcrotonyl-CoA to accumulate in the mitochondria where it reacts with glycine (9) and potentially depletes this amino acid from the mitochondrial matrix.

Succinyl-CoA from the TCA cycle and glycine are the precursors for heme biosynthesis. Heme synthesis starts in the mitochondria by condensing succinyl-CoA with glycine to form

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[2] Abbreviations used: ACC, acetyl-CoA carboxylase; BD, biotin deficient/deficiency; BDC, biotin-dependent carboxylase; BD+B, BD + biotin; BS, biotin-sufficient/sufficiency; BS+B, BS + biotin; cdFBS, FBS stripped by charcoal/dextran; FBS, fetal bovine serum; MCC, 3-methylcrotonyl-CoA carboxylase; PDL, population doubling level; PCC, propionyl-CoA carboxylase; PC, pyruvate carboxylase; TCA, tricarboxylic acid (Krebs) cycle; LMPA, low melting point agarose; DPBS, Dulbecco's PBS; DCFH, 2′, 7′-dichlorodihydrofluorescein.

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δ-aminolevulinate, the first metabolite committed to heme synthesis (10).

We hypothesized that metabolic conditions that interfere with the optimal activity of the TCA cycle may decrease heme synthesis (11). The rationale for our hypothesis is that all the metabolites produced from the TCA cycle intermediates except heme return to the TCA pool of intermediates during catabolism and/or can be supplied from the diet (e.g. amino acids) (8,11). Heme, however, must be synthesized in situ as dietary heme is degraded and does not return to the TCA cycle intermediate pool after catabolism by heme oxygenase (11). Therefore, we proposed that when the activity of the TCA cycle is limited, e.g. in deficiency of biotin, the metabolic burden falls mainly on heme synthesis (11).

Materials and Methods

Materials. Fetal bovine serum (FBS) and FBS stripped by charcoal/dextran (cdFBS) were from Hyclone. PBS, trypsin-EDTA, DMEM, and HEPES (1 mol/L) were from Gibco/Invitrogen. EDTA, Tween-20, biotin, deferoxamine, butyl acetate, and protease inhibitor cocktail were from Sigma-Aldrich. Avidin and HRP-conjugated NeutroAvidin were from Pierce. Iron isotope, as $^{59}FeCl_3$ (1 Ci/L), was from Perkin Elmer, protein quantitation kit was from Bio-Rad, and liquid scintillation CytoScint was from MP Biomedicals (formerly ICN Biomedicals). Hemin was purchased from Frontier Scientific. Low melting point agarose (LMPA) was NuSieve GTG Agarose from BioWhittaker Molecular Applications and CometSlide was from Trevigen.

In vitro model for BD. cdFBS contains 0.29 μg/L biotin, ~0.6% that of normal serum (FBS; 47 μg/L) (12). We confirmed the low biotin content of cdFBS by using ELISA (data not shown). Compared with the marked change in biotin, the other vitamins, hormones, and minerals were unaffected or minimally affected upon treating with charcoal/dextran (12). FBS is the only source for biotin in the medium, because biotin, unlike the other micronutrients, is not supplemented in DMEM. Thus, any minor reductions to micronutrient levels other than biotin were replenished by adding DMEM. We demonstrated that cdFBS-supplemented medium causes BD. Establishing this model allowed us to perform long-term experiments and study the consequences of BD on the mitochondria and cellular senescence. Avidin-conjugated beads from Sigma-Aldrich were also used to create BD (13).

Detection of BDC and specific mitochondrial proteins. The biotinylation status of PC, PCC, and MCC was determined by the avidin blotting technique (14). The cells were harvested by trypsinization and washed twice with the respective medium. Lysate was prepared in 1% Tween 20, antiproteases, and sonicated in ice. Cellular proteins (100 μg) were resolved in 15% SDS-PAGE, transferred to polyvinylidene difluoride membrane, incubated for 1 h with 1.5 μg in 15 mL PBS NeutrAvidin-HRP conjugate, and detected by chemiluminescence and exposure to imaging film. The different components of the mitochondrial electron transport complexes were evaluated by 15% SDS-PAGE and Western blotting using antibodies against selected subunits from complexes I, III, and IV. For complex IV we used subunit II (COX-II), for complex III we used subunit CorI, and for complex I we used ND39. Quantification of the protein bands in the avidin blot or western blot was performed by densitometry analysis of bands detected on the film using ImageJ software (NIH).

In vitro cellular senescence. Primary human lung fibroblasts (IMR90 from Coriell Institute for Medical Research) are an in vitro model for cellular senescence (15). IMR90 are started in culture as young cells with a low population doubling level (PDL) and allowed to increase in PDL until senescence (high PDL). At senescence, the cells are viable and metabolically active, although they have lost replicative capacity.

To test the effect of BD on PDL, the same batch of IMR90 cells were seeded at 0.5×10^6 per 100-mm dish in: *1*) medium supplemented with 10% cdFBS (BD); *2*) medium supplemented with 10% normal FBS [biotin-sufficient (BS)]; *3*) medium supplemented with 10% cdFBS + 5 μg/L biotin [BD + biotin (BD+B)]; and 4) medium supplemented with 10% normal FBS +5 μg/L biotin [BS + biotin (BS+B)]. All the cultures were split after 7 d, and PDL was calculated as $\log_2 (D/D_o)$, where D and D_o are defined as the density of cells at the time of harvesting and seeding, respectively (16). The cells were seeded again in fresh medium as described above. Additional cells from each splitting cycle were collected and stored at −20°C for further analysis.

Extraction of heme for HPLC analysis. Heme was measured using the HPLC column Bond-Clone-C18, 300 × 3.9 mm (Phenomenex), as previously described (17). About 5 million cells lysed into 200 μL ice-cold PBS/1 mmol/L EDTA/ 0.2% Tween 20/protease inhibitors. A total of 20 μL of the lysate was used for heme extraction (17). Heme content was normalized to total protein content (Bio-Rad protein quantification kit).

Measurement of heme synthesis in BD and BS cells. The effect of BD on heme synthesis was tested using the same batch of cells. They were grown on BD, BS, or BD + B (5 or 50 μg/L) media. Heme synthesis was measured using ^{59}Fe, which is incorporated to form heme as described previously (18). The radioactivity incorporated into newly synthesized heme was measured using a liquid scintillator radiation counter. Total protein content in the sample was measured by Bio-Rad protein assay kit and used for normalization of heme synthesis and iron uptake.

Measuring production of oxidants. 2′, 7′-dichlorodihydrofluorescein (DCFH) was used to assay the production of oxidants in cells. IMR90 cells deficient, sufficient, and deficient supplemented for biotin for 3 wk were seeded in 6-well plates with the respective media for 1 wk. The media were removed from the wells and the cells were rinsed with 3 mL Dulbecco's PBS (DPBS). A stock solution of 5 mmol/L DCFH was prepared in ethanol and kept from light. DCFH was added to cells at 25 μmol/L in 3 mL DMEM, incubated at 37°C for 30 min, and followed by 3 washes with 3 mL Hank's DPBS supplemented with magnesium and calcium. Three milliliters of HBSS was added to each well after the final wash. Fluorescence was measured with a CytoFluor 2350 Fluorescent Measuring System plate reader, using 480-nm excitation and emission at 530 nm. For each of the conditions of the cultured cells, a background reading was taken with the procedure described above, omitting the addition of DCFH and including the addition of ethanol. The background reading was subtracted from the actual reading and the fluorescence was normalized to cell number. The cells in each well were counted using a Coulter Counter (Beckman Coulter) after rinsing with DPBS and incubating with trypsin. Results are presented as arbitrary fluorescent units per million cells.

Measuring oxidative damage to DNA by the comet assay. The alkaline (pH >13) comet was performed as previously described (19). IMR90 cells were grown for 2–4 wk then rinsed, trypsinized, and added to 0.6% LMPA at a concentration of 50,000 cells/0.5 mL LMPA. A total of 50 μL of the cell solution was then added to each CometSlide. After lysing, slides were incubated in alkaline electrophoresis solution followed by electrophoresis at 0.74 V/cm for 20 min, not exceeding 300 mA. Following neutralization with 0.4 mol/L Tris, pH 7.5, and dehydration with ethanol and methanol, slides were incubated for 30 min in SYBR Green diluted 1:10000 in TE (0.01 mol/L Tris + 0.001 mol/L EDTA, pH 7.4) buffer. Slides were then viewed with a fluorescent microscope (Zeiss Axiovert 25 Light/Fluorescence Inverted Microscope) and multiple images of cells were captured (Spot Junior Digital Camera). Individual cells were analyzed using CometScore v1.5 (TriTek). The total number of cells examined was >70 cells per treatment. During analysis, the operator was blind for the identity of the samples.

Statistical analysis. Statistical analyses (*t* tests, nonparametric Mann-Whitney tests, or 1-way ANOVA) were performed using Prism 4.0 (GraphPad) software. When appropriate, post hoc Tukey, Bonferroni, or Dunn's tests were conducted. Differences were considered significant at $P < 0.05$. Values in the text are means ± SE.

Results

BD cdFBS medium. Cells grown in cdFBS-supplemented medium lost their BDC (**Fig. 1**), indicating that cdFBS-supplemented medium was BD. Three BDC, PC, PCC, and MCC, were undetectable in cells maintained for 1 to 2 wk in cdFBS-supplemented medium (Fig. 1). PCC and MCC appeared in the avidin blot as a single wide band, because their molecular mass are similar: 76 and 77 kDa, respectively (14,20). Cells grown in biotin-replenished cdFBS medium showed substantial recovery of BDC (Fig. 1), adding further support that cdFBS-supplemented medium is BD. Based on the results in Figure 1 and additional results presented below, we refer to cells maintained in cdFBS-supplemented medium as BD and cells maintained in medium supplemented with FBS as BS. Similar results were obtained when cells were maintained in serum stripped of biotin using avidin-conjugated beads (data not shown).

Cellular senescence. IMR90 cells had fewer cumulative numbers of PDL until cell senescence when maintained in BD medium, compared with BS or BS+B medium, indicating an increased rate of cellular senescence (**Fig. 2***A*). The cells grown in BS medium had a 10.6 gain in PDL compared with cells grown in BD medium (Fig. 2*A*). When the cells were grown in BD+B medium, the cellular senescence did not differ from cells grown in BS medium (Fig. 2*B*), evidence of a cause-and-effect relation between BD and early senescence. Whereas the decrease in BDC started as early as the first week after cells were placed in a BD medium, the effects of BD on PDL were not detectable during the first 2 to 3 wk of the experiment (Fig. 2*B*). This observation suggests that the consequence of BD occurs slowly and cumulatively. Furthermore, an intracellular mechanism may exist that enables young cells to resist the consequences of BD more than old cells, possibly by compensatory upregulation of important, metabolic, salvage, and repair mechanisms.

Cellular heme content. A 36% decrease in the heme level occurred in cells grown in BD for 2–3 wk (from 38.2 ± 2.4 ng/mg protein in BS to 24.1 ± 1.7 ng/mg protein in BD, $P = 0.005$, $n = 3$). A 50% decrease in heme occurred in cells grown in BD for 6 wk (from 27.6 ± 9 ng/mg protein in BS to 12.0 ± 8.5 ng/mg protein in BD, $P = 0.03$, $n = 6$). Adding biotin back to the medium restored the heme content to 24.5 ± 3.9 ng/mg protein. The difference in heme levels between the 2 control cell culture likely was due to the different batches of cells used.

Cellular heme synthesis. The rate of heme synthesis in IMR90 cells maintained in BS medium was higher than IMR90 cells maintained in BD medium by 5.21 ng heme mg protein$^{-1} \cdot 3\ h^{-1}$ ($n = 10$, **Fig. 3***A*). Cells maintained in BD medium supplemented with 5 or 50 μg/L biotin synthesized heme at ~77 and 93% of the capacity of BS cells, respectively (Fig. 3*A*). Because a change in heme synthesis requires cellular uptake of iron, the changes in iron uptake paralleled those of heme synthesis (Fig. 3*B*). Iron uptake was restored upon adding biotin back to the growth medium.

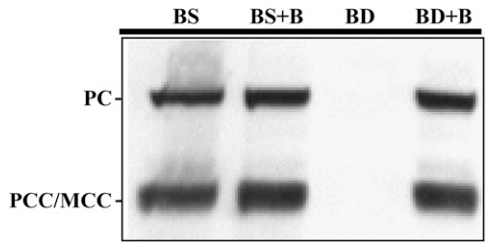

Figure 1 BDC are not detectable in IMR90 cells maintained in BS, BD, BS+B, and BD+B media for 3 wk. PCC and MCC differ by ~1 kDa and appear as a single broad protein band. Shown is 1 representative experiment of 6.

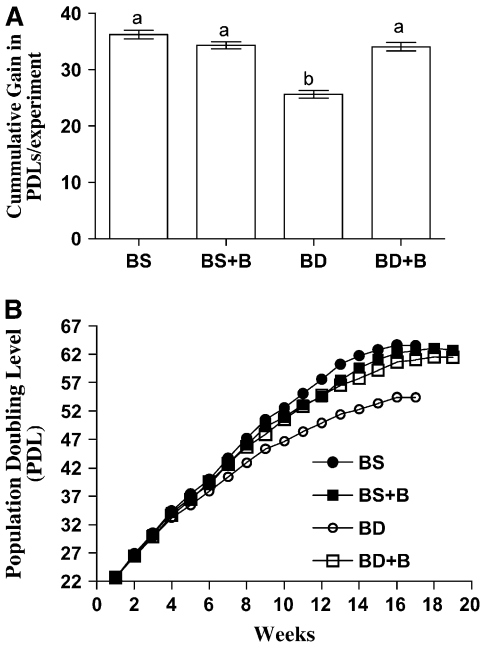

Figure 2 Senescence of IMR90 cells maintained in BS, BD, BS+B, and BD+B media. Senescence of IMR90 was determined by calculating the weekly gain in PDL. The cumulative gain in PDL of IMR90 cells from the start until the end of the 16- to 20-wk experiments (*A*). Values are means ± SE, $n = 6$. Means without a common letter differ, $P < 0.05$ (ANOVA, Tukey's test). One representative experiment for IMR90 senescence of 6 shown in *A* (*B*). Young cells (low PDL) were split every week, the PDL calculated, and a portion of the cells reseeded. This procedure was repeated until the cells senesced (i.e. no increase in PDL).

Mitochondrial complex IV. Because the TCA cycle is dependent on biotin availability, we measured the levels of mitochondrial complexes I, III, and IV to assess the status of electron transport chain complexes of the mitochondria in cells following 1–2 wk of BD. BD cells had a lower level of complex IV than BS cells, whereas mitochondrial complexes I and III did not differ (**Fig. 4**).

We compared the kinetics of complex IV's decline after inducing BD in old and young cells. Exposure to short periods of BD (24–72 h) caused an ~50% decline in complex IV in old cells within 24 h ($P < 0.02$) and 48 h ($P < 0.0001$) of introducing the deficiency. The decline in complex IV in senescent cells due to BD exceeded that in younger cells (**Fig. 5**). When the BD persisted, complex IV decreased to almost undetectable levels, as in Figure 4.

Like heme-*a*, copper is a structural component in complex IV. Copper is ~57% lower in cdFBS and is not included in DMEM. To exclude the possibility that copper contributes to the consequences of BD, we tested whether restoring copper to levels in FBS affects the results of BD. Adding copper did not affect heme synthesis or cellular senescence, suggesting the amount of copper in the cdFBS is enough to sustain normal metabolism of the cells. However, as expected from copper's role in complex IV, the recovery of complex IV was more efficient when copper and biotin were both added to the medium (data not shown).

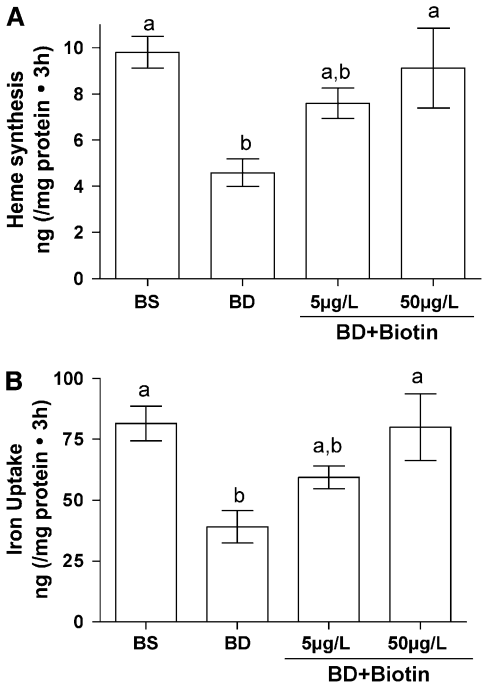

Figure 3 Heme synthesis (*A*) and iron uptake (*B*) in IMR90 cells maintained in BS, BD, or BD+B (5 or 50 μg/L) media for 3 wk. Values are means ± SE, $n = 8$. Means without a common letter differ, $P < 0.05$ (ANOVA, Tukey's test).

Oxidant production and DNA damage. The rate of DCFH oxidation in BD cells was twice as high as in BS cells, indicating an increase in the production of oxidants (**Fig. 6***A*). Adding biotin back to the growth medium decreased the rate of oxidant production to that of BS cells. DNA damage in BD cells, measured by comet tail moment, was significantly higher than in BS cells, indicating an increase in oxidation of DNA (Fig. 6*B*). Returning biotin to the growth medium decreased the rate of oxidative damage to DNA to the rate in BS cells.

Discussion

This study established a key role for biotin in maintaining mitochondrial complex IV, heme metabolism, and preventing oxidative damage to DNA in human primary fibroblasts. The metabolic consequences of BD were prevented by returning biotin to the growth medium. The mechanism responsible for these effects of BD likely involves biotin's role in the TCA cycle. The concentration of biotin in BD medium is ~100 pmol/L, and in human plasma, ~250 pmol/L; how these correspond needs to be studied.

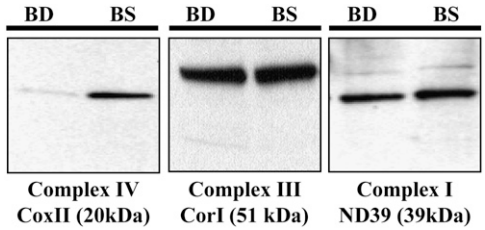

Figure 4 Levels of mitochondrial complex IV in IMR90 cells maintained in BS or BD medium for 1–2 wk. Proteins were separated by 15% SDS-PAGE and Western blotted to a polyvinylidene difluoride membrane. The membrane was incubated with antibodies specific for complexes IV (COX-II), I (ND39), and III (CorI). A representative gel is shown.

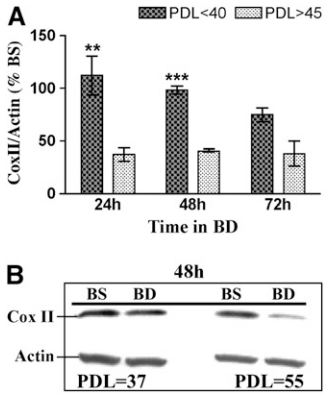

Figure 5 Differential responses to BD in old (PDL >45) and young (PDL <40) IMR90 cells maintained in BS or BD medium for 24, 48, and 72 h. Quantitative representation of the density of the bands in western blots for complex IV similar to that shown in *B* (48-h incubation) (*A*). The band density of COX-II was normalized to actin. Values are means ± SD, $n = 3$. Asterisks indicate different from PDL <40: $^{**}P < 0.02$, $^{***}P < 0.0001$ (Mann-Whitney).

BD accelerates cellular senescence in vitro (21) (Fig 2). Mitochondria have been proposed to play a key role in cellular senescence and aging (11,22). Therefore, key biochemical variables of mitochondria were assayed. BD caused the loss of mitochondrial complex IV but did not affect the mitochondrial complexes I and III (Fig. 4). Selective loss of complex IV increases the ratio of complexes I and III to complex IV and also increases the cellular oxygen concentration. Complex IV is the main oxygen-metabolizing enzyme; it converts >95% of cellular oxygen to water. Deficiency of complex IV is known to increase the production of oxidants by mitochondria (23). Consistent with this, we found that the levels of oxidants and oxidative damage to DNA were greater in BD cells compared with BS cells (Fig. 6); these alterations may contribute to the acceleration of

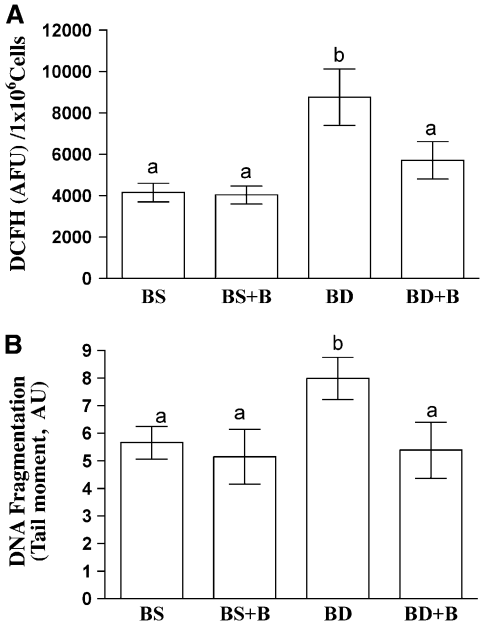

Figure 6 Oxidants produced (*A*) and DNA fragmentation (*B*) in IMR90 cells maintained in BS, BD, BS+B, or BD+B media for 3 wk (*A*) or 2–4 wk (*B*). *A*: Values are means ± SE, $n = 6$. Means without common letter differ, $P < 0.05$ (ANOVA, Bonferroni test). *B*: Values are means ± SE, $n = 6$. Means without common letter differ, $P < 0.05$ (ANOVA, Dunn's test).

cellular senescence by BD (Fig. 2). We have previously demonstrated that the antioxidant N-*tert*-butyl hydroxylamine prevents cdFBS-induced early senescence (21).

Heme level and synthesis were markedly decreased in BD cells (Fig. 3), indicating that adequate heme synthesis requires biotin and that BD can cause heme deficiency. Thus, biotin should be considered the 8th member of the group of vitamins and minerals required for adequate heme synthesis (11). The decrease in iron uptake in BD cells (Fig. 3) is unexpected, because heme deficiency should be expected to cause a compensatory increase in iron uptake (24,25). A possible explanation for the lack of an increase in iron uptake in BD cells is that the heme deficiency caused by BD is due to a decrease in succinyl-CoA, which lowers the production of porphyrins. Porphyrins are intermediates in the biosynthesis of heme. These results suggest that optimal uptake of iron requires that the mechanisms for iron assimilation into heme remain intact. Adequate levels of biotin appear to be essential for adequate iron uptake. Thus, for correcting iron deficiency in humans, it may be important to ensure biotin adequacy.

Heme is essential in intermediary metabolism, gene regulation, and mitochondrial integrity (26). In this study, we used the decrease in complex IV in response to BD as a functional indicator of heme deficiency (Fig. 4). We previously demonstrated that heme deficiency causes selective inactivation of mitochondrial complex IV (27,28). A likely reason for this effect is that heme is a precursor for heme-*a*. Heme-*a* is a unique type that exists only in complex IV. The maturation of heme to heme-*a* requires important biochemical modifications (29). Thus, a shortage in heme causes a decrease in heme-*a*, leading to a selective decrease in mitochondrial complex IV. Complex IV is an enzyme complex made of 13 subunits (e.g. subunits COX-I, COX-II, COX-III, etc.) (30). The assembly of complex IV starts with the assimilation of heme-*a* into COX-I, followed by assembly of COX-IV&II, then the rest of the subunits. Thus, the assembly of the entire complex depends on proper folding of COX-I (31,32), which becomes limiting in heme deficiency. We used subunit COX-II as representative of the entire complex (28). With the exception of complex IV, heme proteins, such as complex III, use heme directly and have no need for maturation. A selective decline in complex IV triggers the production of oxidants and free radicals by the mitochondria (27,28). Therefore, it is likely that heme deficiency is the mechanism by which BD causes selective loss of complex IV, mitochondrial decay, and oxidative stress and accelerates cellular senescence.

A limitation in the supply of TCA cycle intermediates may also decrease the production of ATP and limit the utilization of energy from biochemical sources. This change in energy metabolism may also contribute to the metabolic consequences of heme deficiency. We, and others, have demonstrated that low levels of heme do not immediately decrease cell viability, but rather low levels disrupt cellular responses to stress, block differentiation, and alter intermediary metabolism (27,33,34).

Old cells are more susceptible to BD than young cells (Fig. 5). This finding is consistent with old cells being slower than young cells to recover from heme deficiency (28). We therefore propose that mitochondria from old tissues are more susceptible to BD and heme deficiency (28). An age-dependent elevation in biotin absorption has been shown in old rats (7). In humans, plasma biotin concentrations appear to increase with age (35). Because plasma biotin does not always reflect tissue biotin (36), this increase may indicate a change in biotin metabolism with age. More research is needed to determine the relevance of these findings to the biology of aging in humans.

The mechanistic links among biotin, the TCA cycle, the integrity of heme metabolism, and mitochondria may provide in part an explanation for the teratogenic consequences of BD in rodents (2). The requirement of biotin for the metabolic integrity of the developing fetus appears high (4), probably due to rapid growth and increase in mass. Recent studies showed that proliferation of lymphocytes consumes large amounts of biotin (37), which might also be true for other types of cells. Therefore, the teratogenic consequences of reduced availability of biotin may be triggered in part by reduced mitochondrial function, abnormal heme metabolism, and abnormal histone biotinylation. All of these metabolic activities are likely to be critical for normal development.

The effect of biotin on glucose status in diabetes mellitus patients may be a result of improved mitochondrial function, particularly the TCA cycle (38,39). The TCA cycle may also fail to supply enough succinyl-CoA when there are deficiencies of the vitamins that are essential for the production of TCA cycle intermediates. A combination of deficiencies and exposure to toxins that require increased heme synthesis would aggravate the cell's need for succinyl-CoA (11). The importance of biotin in heme synthesis and maintaining the TCA cycle warrants further investigation to the extent of BD as well as the optimal levels of biotin needed by the population.

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