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Clusters of biotin-responsive genes in human peripheral blood mononuclear cells

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Abstract

Effects of biotin in cell signaling are mediated by transcription factors such as nuclear factor–κB (NF-κB) and Sp1/Sp3 as well as by posttranslational modifications of DNA-binding proteins. These signaling pathways play roles in the transcriptional regulation of numerous genes. Here we tested the hypothesis that biotin-dependent genes are not randomly distributed in the human genome but are arranged in clusters. Peripheral blood mononuclear cells were isolated from healthy adults before and after supplementation with 8.8 μmol/day biotin for 21 days. Cells were cultured *ex vivo* with concanavalin A for 3 hours to stimulate gene expression. Abundances of mRNA encoding ~14,000 genes were quantified by both DNA microarray and reverse transcriptase–polymerase chain reaction. The expression of 139 genes increased by at least 40% in response to biotin supplementation, whereas the expression of 131 genes decreased by at least 40% in response to biotin supplementation. The following clusters of biotin-responsive genes were identified: 1) 16% of biotin-responsive gene products localized to the cell nucleus; at least 28% of biotin-responsive genes play roles in signal transduction (these findings are consistent with a role for biotin in cell signaling); and 2) of the biotin-responsive genes, 54% clustered on chromosomes 1, 2, 3, 11, 12, and 19, whereas no biotin-responsive genes were found on chromosomes 10, 16, 18, 21, and heterosomes. This suggests that position effects play a role in biotin-dependent gene expression. Collectively, these findings suggest that the human genome contains clusters of biotin-dependent genes.

Keywords: Biotin; DNA microarrays; Gene expression; Human; Peripheral blood mononuclear cells

1. Introduction

In mammals, biotin serves as a covalently bound coenzyme for acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase [1]. These enzymes catalyze essential steps in the metabolism of glucose, amino acids, and fatty acids [1]. Consistent with the essential roles of these macronutrients in metabolism, biotin deficiency may cause decreased rates of cell proliferation [2,3], impaired immune function [4–6] and abnormal fetal development [7–9].

Evidence has been provided that biotin might also play a role in the expression of various genes in mammals. For example, the expression of genes coding for glucokinase [10–14], holocarboxylase synthetase [15,16], 3-methylcrotonyl-CoA carboxylase [17], biotin transporters [3,18], various cytokines [17,19], and interleukin-2 receptor–γ [19] correlates with biotin supply in humans, human cell lines, and rats. In contrast, biotin supplementation causes decreased expression of genes coding for phosphoenolpyruvate carboxykinase in liver from diabetic rats [20] and interleukin-4 in peripheral blood mononuclear cells (PBMC) from healthy adults [17]. Theoretically, abnormal gene expression might account for some of the adverse effects of biotin deficiency in humans and animals.

Evidence has been provided that the following signals play a role in the regulation of biotin-dependent genes. The nuclear translocation of nuclear factor–κB (NF-κB) is greater in biotin-deficient compared with biotin-supplemented T cells, mediating increased expression of NF-κB–dependent genes in biotin-deficient cells (Rodriguez-Melendez R, Schwab LD, Zempleni J; submitted for publication). In contrast, nuclear translocation of transcription factors Sp1 and Sp3 is greater in biotin-supplemented compared with biotin-deficient T cells [21]. Sp1 may act as transcrip-

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tional activator for numerous genes, whereas Sp3 may act as transcriptional activator or repressor [22]. Finally, an enzymatic mechanism has been proposed that mediates covalent binding of biotin to histones (DNA-binding proteins) [23]; biotinylated histones have been detected in various mammalian cell lines [3,18,24,25]. Covalent modifications of histones play important roles in regulating transcription, replication, and repair of DNA [26].

Theoretically, biotin-dependent cell signals may converge on clusters of genes that are defined by denominators such as chromosomal localization and molecular function. These clusters can be identified by DNA microarrays, which contain probes for a large fraction of the human transcriptome. Here we conducted a DNA microarray analysis to test the hypothesis that biotin-dependent genes are not randomly distributed in the human genome but are arranged in clusters.

2. Methods and materials

2.1. Subjects

Eight healthy adults aged 25–51 years participated in this study; one man and four women were of white ethnicity, two women were Hispanic, and one woman was Asian. All subjects were nonsmokers; none had knowingly consumed any vitamin supplements for at least 3 weeks before initiation of the study. Pregnant women and individuals treated with anticonvulsants were not eligible for study participation [1]. This study was approved by the Institutional Review Board at the University of Nebraska–Lincoln.

2.2. Study design

A heparinized blood sample (~150 mL) was collected from each subject before biotin supplementation (denoted "presupplementation"). A second blood sample (denoted "postsupplementation") was collected approximately 2 hours after subjects had completed a 21-day supplementation with biotin; one pill of Solaray Biotin (Solaray, Inc., Park City, UT) was taken daily. Biotin content of the supplement was determined by avidin-binding assay as described previously [27] with modifications [3]; biotin content was 8.8 μmol/pill (2150 μg). The normal dietary intake of biotin is approximately 0.4 μmol/day [28]. Previous studies have suggested that 3 wk of biotin supplementation are sufficient to achieve new steady-state levels of biotin in immune cells [29].

2.3. Cell culture

PBMC were isolated aseptically from blood by gradient centrifugation [30]; plasma was saved for determination of biotin concentration and for use as a culture supplement. PBMC (approximately 3.5×10^9 cells/L) were suspended in biotin-defined RPMI-1640 (Atlanta Biologicals, Norcross, GA), supplemented with autologous plasma, penicillin, and streptomycin [17]. Antibiotics and culture medium from the same stock solutions and powder, respectively, were used in all experiments. For cell cultures, we attempted to simulate the likely plasma levels of biotin before and during supplementation of subjects. The biotin concentration in culture media was adjusted to 0.25 nmol/L for presupplementation PBMC and to 10 nmol/L for postsupplementation PBMC, based on plasma concentrations of biotin plus biotin metabolites observed in our previous studies [31]. Concanavalin A at a final concentration of 20 mg/L was added to culture media immediately after suspending the cells to stimulate gene expression; concanavalin A from the same stock solution was used in all experiments. At 3 hours after stimulation, cells were collected by centrifugation ($250 \times g$ for 10 minutes) for isolation of RNA.

2.4. Plasma biotin

Biotin and biotin metabolites in plasma were quantified by avidin-binding assay [3,27]. It is noteworthy that avidin-binding assays are not absolutely specific for biotin but may also bind biotin metabolites and other compounds [32]. Thus, avidin-binding assays are likely to overestimate the concentration of biotin in plasma.

2.5. Isolation of RNA

Total RNA was extracted from PBMC by using the RNeasy mini kit and the RNase-free DNase set (Qiagen, Valencia, CA). Absence of contaminations with genomic DNA was confirmed by polymerase chain reaction (PCR) using primers specific for intronic sequences of the interleukin-2 gene [17].

Absorbances at 260 and 280 nm were measured for each RNA sample; the absorbance ratio (260–280 nm) was 1.9 ± 0.2, consistent with the absence of significant contamination with protein. To confirm integrity of RNA, 22 μg of RNA was electrophoresed using a formaldehyde–agarose gel [15]; RNA was stained with ethidium bromide and visualized using a Kodak EDAS 290 Documentation and Analysis System (Eastman Kodak, Rochester, NY). Two major ribosomal bands (28S and 18S rRNA), but no degraded RNA were detected (data not shown).

2.6. DNA microarrays

Equal amounts of RNA from all eight subjects were pooled to produce two samples: presupplementation and postsupplementation RNA. The cDNA was synthesized from 15 μg of RNA using an OligodT_{24} primer containing the 5′-T7 RNA polymerase promoter sequence (Affymetrix, Santa Clara, CA) and the SuperScript II kit (Invitrogen, Carlsbad, CA). The resulting cDNA was transcribed in vitro using a BioArray High Yield RNA Transcript Labeling Kit

(Enzo Biochem, New York, NY) in the presence of biotinylated UTP and CTP to produce biotinylated target complementary RNA (cRNA). The cRNA was purified, fragmented, and hybridized to the Human Genome U133A Array according to protocols provided by the manufacturer (Affymetrix) in a Hybridization Oven model 640 (Affymetrix). The arrays were washed and stained with streptavidin-phycoerythrin using a GeneChip Fluidics Station model 400 and then scanned using an Agilent Technologies GeneArray Scanner.

2.7. Microarray data normalization and analysis

The Microarray Suite 5.0, Micro DB, and Data Mining Tool 3.0 software (Affymetrix) were used for normalization and analysis of microarray data. The signal value of the experimental array was multiplied by a normalization factor to make its mean intensity equivalent to the mean intensity of the control array, using Microarray Suite software according to the manufacturer's protocol. The absolute call (present, marginal, absent) and average difference of ~14,000 gene expressions in a sample; and the absolute call difference, percent change, and average difference of gene expressions between samples were identified using the above-mentioned software. The genes showing altered expression were categorized on the basis of their cellular component, chromosomal localization, and reported or suggested molecular and biological functions using OntoExpress [33]. Genes that were not annotated or not easily classified were excluded from the functional clustering analysis.

2.8. Reverse transcriptase–polymerase chain reaction

Expression profiles of selected genes were also quantified by reverse transcriptase–polymerase chain reaction (RT-PCR), using the RNA prepared for DNA microarrays. For RT-PCR we selected both genes that were up-regulated and genes that were down-regulated in response to biotin supplementation (as judged by DNA microarray). Abundance of mRNA encoding these genes was assayed at least twice by RT-PCR [34] using the customized primers (Integrated DNA Technologies, Coralville, IA) listed in Table 1. The expression of β-actin was not affected by biotin and was used as control. PCR products were collected at timed intervals for up to 34 PCR cycles, and nucleic acids were quantified by gel densitometry as previously described [17]. Only those values from within the exponential phase of PCR amplification (typically up to 28 PCR cycles) were considered for data analysis.

3. Results

3.1. Plasma biotin

The concentration of biotin plus biotin metabolites was 21 ± 12.2 times greater (range 5.9–39) in postsupplementation plasma (28 ± 19 nmol/L) compared with presupplementation plasma (1.5 ± 0.5 nmol/L; $P < 0.05$), suggesting that subjects complied with the supplementation protocol.

Table 1
Oligonucleotide primers used for reverse transcriptase polymerase chain reaction

Gene	Primer
TNF15	5′-ACCAAGTCTGTATGCGA-3′
	5′-ATATTTAGAAACTCGCC-3′
TUP1	5′-AGTAATGAACCTGTCCGAT-3′
	5′-TATTTCTCGAAGTCGGTAT-3′
LAMG	5′-ATGAACAACGTGACCGTAA-3′
	5′-ACTTGCATTCATCCCGAACT-3′
TPR-28	5′-TCTCAGAAAAGTAAACGTG-3′
	5′-TTACTAGATGCTGGTTACG-3′
Charot	5′-CTGCCTCTTTGTCTACTGGT-3′
	5′-ATATCTCTCCACACTTGCAC-3′
CYP1B1	5′-AAAGTACAACTAACGCAACC-3′
	5′-CCAACTCTTGTCACCTCGTA-3′
β-Actin	5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′
	5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′

Charot = Charot-Leyden crystal protein; CYP1B1 = cytochrome P450 1B1; LAMG = lysosome-associated membrane glycoprotein; TNF15 = tumor necrosis factor ligand superfamily member 15; TPR-28 = nucleoprotein TPR-28; TUP1 = TUP1-like enhancer of split gene.

GenBank accession numbers: β-actin = NM_001101; Charot-Leyden crystal protein = NM_001828; cytochrome P450 1B1 = NM_000104; lysosome-associated membrane glycoprotein = J03263; tumor necrosis factor ligand superfamily member 15 = NM_005118; nucleoprotein TPR-28 = AK023111; TUP1-like enhancer of split gene = X75295.

3.2. Gene expression analysis

Biotin supplementation affected the expression of 270 genes in PBMC as judged by DNA microarray analysis. Genes were considered to be nonresponsive to biotin if expression changed by <40% in response to biotin supplementation. The expression of 139 genes increased by >40% in response to biotin supplementation, whereas the expression of 131 genes decreased by >40% in response to biotin supplementation. The following genes exhibited the greatest increase in response to biotin supplementation: serine protease/enterokinase (885% greater post- *versus* pre-supplementation; GenBank NM_002772), immunoglobulin μ chain antibody MO30 (392% greater post-supplementation; GenBank AI858004), nucleoprotein TPR (225% greater post-supplementation; GenBank AK023111), cytochrome P450 1B1 (164% greater post-supplementation; GenBank NM_000104), and DKFZP564G092 protein (146% greater post-supplementation; GenBank NM_015601). The following genes exhibited the greatest decrease in response to biotin supplementation: DKFZp564D113 (428% greater pre- *versus* post-supplementation; GenBank AL049250), interferon stimulated T-cell α chemoattractant precursor (329% greater pre-supplementation; GenBank AF030514), α chemokine H174 (248% greater pre-supplementation; GenBank AF002985), TUP1-like enhancer of split gene 1

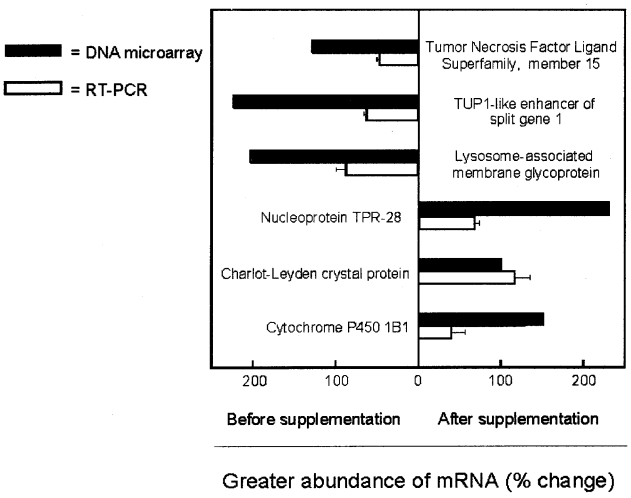

Fig. 1. Comparative analysis of gene expression by DNA microarrays and reverse transcriptase–polymerase chain reaction (RT-PCR). Healthy adults received supplementation with 8.8 μmol biotin/day for 21 days. Peripheral blood mononuclear cells were isolated before and after supplementation with biotin. RNA was isolated from cells 3 hours after stimulation with concanavalin A *ex vivo*. Abundance of mRNA was quantified by DNA microarrays and RT-PCR.

(224% greater pre-supplementation; GenBank X75296), and lysosome-associated membrane glycoprotein lamp A (203% greater pre-supplementation; GenBank J03263).

Were DNA microarray data confirmed by alternative assay procedures? Six biotin-responsive genes were selected from microarray experiments; the abundance of mRNA encoding these genes was quantified by RT-PCR. Comparative analysis of gene expression by DNA microarrays and RT-PCR produced similar trends (Fig. 1); both analytical procedures consistently identified the same genes to be either up- or down-regulated by biotin supplementation. However, the apparent effects of biotin on gene expression were smaller if judged by RT-PCR compared with DNA microarrays. On average, RT-PCR underestimated (or DNA microarrays overestimated) effects of biotin on gene expression by approximately 50% (Fig. 1). Notwithstanding this difference in the magnitude of observed effects, the RT-PCR experiments provide evidence that data obtained by DNA microarrays are reliable. Abundance of mRNA encoding β-actin (control) was not affected by biotin (data not shown).

3.3. Gene clusters

Biotin-dependent genes were not randomly distributed in the genome but could be assigned to gene clusters in human PBMC. If biotin-dependent genes were classified based on the cellular localization of gene products, a significant fraction of these products (16%) localized to the cell nucleus (Table 2). Specifically, 13% of gene products that were down-regulated by biotin localized to the nucleus, whereas 18% of gene products that were up-regulated by biotin localized to the nucleus. This is consistent with roles for biotin-dependent genes in signal transduction as described below. Another important cluster of gene products localized to membrane-associated compartments in PBMC. The various membrane compartments combined contained 26% (70 genes) of the biotin-dependent gene products (Table 2). Gene products of unknown localization and cellular compartments with fewer than four entries are not listed in Table 2.

Of the biotin-responsive genes, 54 clustered on chromosomes 1, 2, 3, 11, 12, and 19, whereas no biotin-responsive genes were found on chromosomes 10, 16, 18, 21, and heterosomes. This suggests that position effects might play a role in biotin-dependent gene expression; these effects might be mediated by biotinylation of DNA-binding proteins (see Discussion).

Classification of biotin-responsive genes by the categories of “molecular function” and “biological process” provided evidence that effects of biotin in cell signaling might be mediated by transcriptional activity of genes that play roles in signal transduction. For example, the following clusters of biotin-responsive genes were identified in the molecular function category (Table 2): gene products that have ATP/protein-binding activity (5% of biotin-responsive genes), DNA/protein-binding activity (4%), transcription factor activity (4%), RNA binding activity (4%), chemokine activity (3%), and receptor activity (3%). These gene products play roles in cell signaling. Clusters of genes of unknown molecular function and clusters of genes with fewer than three entries are not listed in Table 2.

Clusters of biotin-responsive genes in the biological process category are consistent with the hypothesis that effects of biotin in cell signaling are mediated by transcriptional activity of genes that play roles in signal transduction. For example, the following clusters of biotin-responsive genes were identified (Table 2): gene products that play roles in signal transduction (14% of biotin-responsive genes), regulation of transcription (7%), cell–cell signaling (4%), and cell surface/receptor-linked signal transduction (3%). Other clusters such as “immune response” and “cell proliferation” may also play roles in signal transduction. Clusters of genes of unknown function in biological processes and clusters of genes with fewer than three entries are not listed in Table 2.

4. Discussion

This study provides evidence that the following are true: 1) biotin supplementation affects expression of genes in human PBMC; 2) biotin-dependent genes are not randomly distributed in the genome but are arranged in clusters; 3) chromosome position effects might play a role in biotin-dependent gene expression; and 4) effects of biotin in cell signaling might be mediated by expression of genes that play roles in signal transduction.

The following mechanisms are likely to mediate the

Table 2
Classification of genes with decreased or increased expression in human peripheral blood mononuclear cells after supplementation of healthy adults with biotin

Cellular Compartment			Chromosomal Localization		
	Decrease	Increase		Decrease	Increase
Nucleus	17	25	Chromosome 1	12	20
Integral to membrane	11	10	Chromosome 2	14	12
Integral to plasma membrane	9	14	Chromosome 4	13	9
Cytoplasm	9	4	Chromosome 5	9	9
Membrane	8	6	Chromosome 6	5	10
Membrane fraction	6	6	Chromosome 7	6	5
Extracellular space	4	8	Chromosome 8	4	5
			Chromosome 9	6	3
			Chromosome 11	7	14
			Chromosome 12	12	11
			Chromosome 13	4	5
			Chromosome 14	9	10
			Chromosome 15	4	5
			Chromosome 17	9	9
			Chromosome 19	12	9
			Chromosome 22	5	3

Molecular Function			Biological Process		
	Decrease	Increase		Decrease	Increase
ATP/protein-binding activity	7	6	Signal transduction	23	15
Protein transporter activity	7	4	Immune response	14	10
Transferase activity	6	6	Regulation of transcription (DNA-dependent)	7	11
DNA/protein-binding activity	6	5			
Transcription factor activity	6	5	Inflammatory	7	5
Protein-binding activity	6	3	Cell-cell signaling	7	5
Hydrolase activity	4	10	Regulation of cell cycle	7	4
Chemokine activity	4	3	Cell proliferation	6	5
RNA binding activity	3	8	Chemotaxis	5	5
Electron transporter activity	3	6	Cell surface/receptor-linked signal transduction	3	5
Receptor activity	3	6			
Tumor suppressor	3	3	Protein/amino acid transport phosphorylation	3	5
			Electron transport	3	4
			Cell motility	3	3

Gene products were classified by cellular compartment, chromosomal localization, molecular function, and biological process. “Decrease” and “Increase” denote decreased and increased, respectively, expression of genes in peripheral blood mononuclear cells after supplementation of healthy adults with 8.8 μmol biotin/day for 21 days.

formation of biotin-dependent gene clusters. First, binding of biotin to histones might affect transcriptional activity of individual genes or regions in the genome. Biotinylated histones are enriched in transcriptionally inactive chromatin, suggesting a role for biotinylation of histones in gene silencing [25]. There is precedence for enrichment of various histone modifications in transcriptionally silent heterochromatin and transcriptionally active euchromatin. For example, histone H3 methylated at lysine 9 is strictly localized to heterochromatic intervals in fission yeast; these silent regions are flanked by inverted repeats that mark boundary elements [35]. Hypo-acetylated histones are also enriched in silent chromatic domains [36,37]. This is consistent with a role for (hyper-)biotinylation of histones in gene silencing [25], given that biotin and acetate compete for binding to the same sites in histones [38]. Biotinylated histones have been identified in various human [18,39] and avian cells [25], including PBMC and T cells [3,24,25].

Second, evidence has been provided that biotin affects the nuclear abundance of transcription factors NF-κB (Rodriguez-Melendez R, Schwab LD, Zempleni J; submitted for publication), Sp1, and Sp3 [21]. Nuclear translocation of NF-κB is greater in biotin-deficient immune cells compared with biotin-sufficient controls; this effect is mediated by accelerated breakdown of inhibitors of NF-κB in biotin-deficient cells (Rodriguez-Melendez R, Schwab LD, Zempleni J; submitted for publication). Nuclear abundance of Sp1 and Sp3 is greater in biotin-sufficient immune cells compared with biotin-deficient cells; this effect is mediated by increased transcriptional activity of genes encoding Sp1 and Sp3 [21]. NF-κB, Sp1, and Sp3 affect the expression of a large number of genes, mediating some of the effects of

biotin on gene expression. Information regarding roles for other transcription factors in biotin-dependent gene expression is scarce. Apparently, the nuclear abundance of Oct-1 [21] and P.U.1 (unpublished observation) is not affected by biotin, as judged by electrophoretic mobility shift assay. Likewise, the transcriptional activity of genes encoding the transcription factors CRE and AP-1 does not depend on biotin, as judged by using reporter-gene constructs (unpublished observation).

Evidence has been provided that biotin catabolites may also have biotin-like effects regarding gene expression. Supplementation of culture media with the biotin analogs diaminobiotin and desthiobiotin enhances the transcriptional activities of genes encoding interleukin-2 and interleukin-2 receptor γ in immune cells; the magnitude of these effects is similar if medium is supplemented with biotin [40]. Moreover, supplementation of culture media with the naturally occurring biotin catabolite bisnorbiotin is associated with a unique pattern of gene expression in human HepG2 hepatocarcinoma cells (Rodriguez-Melendez R, Griffin JB, Zempleni J; manuscript in preparation). These observations suggest that some of the effects of biotin supplementation observed in the present study might be caused by biotin catabolites by yet unknown mechanisms.

The following observation is consistent with the hypothesis that effects of biotin on gene expression are physiologically important. The present study provides evidence that biotin supplementation causes a substantial increase in the expression of the gene encoding cytochrome P450 1B1 (CYP1B1). Human cytochromes P450 are a superfamily of 50 genes and 58 pseudogenes [41]. Cytochromes P450 hydroxylate xenobiotics and estrogens ("metabolic activation"), creating electrophilic mutagens [42]. For example, CYP1B1 activates procarcinogens and promutagens such as polycyclic aromatic hydrocarbons and aryl amines [43], enhancing the susceptibility for lymphomas [44]. Likewise, CYP1B1 hydroxylates endogenous 17β-estradiol [45]; 4-hydroxylated estradiol causes single-strand breaks of DNA and 8-hydroxylation of guanine bases in DNA [46,47]. Metabolic activation of carcinogens and estrogens is a key factor in cancerogenesis of the human prostate [48,49], breast [46], and uterus [50]. CYP1B1 is expressed in most peripheral tissues and in malignant tumors [43,51,52]. These observation suggest that supplementation of healthy adults with biotin might be associated with increased metabolic activation of procarcinogens and promutagens by CYP1B1. This hypothesis is currently being tested in our laboratory.

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