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Biotin supplementation decreases the expression of the *SERCA3* gene (*ATP2A3*) in Jurkat cells, thus, triggering unfolded protein response☆

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Abstract

Protein folding in the endoplasmic reticulum (ER) depends on Ca^{2+}; uptake of Ca^{2+} into the ER is mediated by sarco/endoplasmic reticulum Ca^{2+}-ATPase 3 (SERCA3). The 5′-flanking region of the *SERCA3* gene (*ATP2A3*) contains numerous binding sites for the transcription factors Sp1 and Sp3. Biotin affects the nuclear abundance of Sp1 and Sp3, which may act as transcriptional activators or repressors. Here we determined whether biotin affects the expression of the *SERCA3* gene and, thus, protein folding in human lymphoid cells. Jurkat cells were cultured in media containing 0.025 nmol/L biotin (denoted "deficient") or 10 nmol/L biotin ("supplemented"). The transcriptional activity of the full-length human SERCA3 promoter was 50% lower in biotin-supplemented cells compared to biotin-deficient cells. Biotin-dependent repressors bind to elements located 731–1312 bp upstream from the transcription start site in the *SERCA3* gene. The following suggest that low expression of SERCA3 in biotin-supplemented cells impaired folding of secretory proteins in the ER, triggering unfolded protein response: (i) sequestration of Ca^{2+} in the ER decreased by 14–24% in response to biotin supplementation; (ii) secretion of interleukin-2 into the extracellular space decreased by 75% in response to biotin supplementation; (iii) the nuclear abundance of stress-induced transcription factors increased in response to biotin supplementation; and (iv) the abundance of stress-related proteins such ubiquitin activating enzyme 1, *growth arrest and DNA damage 153* gene, X-box binding protein 1 and phosphorylated eukaryotic translation initiation factor 2α increased in response to biotin supplementation. Collectively, this study suggests that supplements containing pharmacological doses of biotin may cause cell stress by impairing protein folding in the ER.

Keywords: Biotin; Calcium; Human; Jurkat cells; Protein folding

1. Introduction

Biotin has the following biological functions in mammals. First, biotin serves as a coenzyme for acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase (PCC) and 3-methylcrotonyl-CoA carboxylase [1]. These carboxylases catalyze steps in the metabolism of glucose, amino acids and fatty acids [1]. Second, histones (DNA-binding proteins) contain covalently bound biotin [2]; biotinylation of histones might play a role in gene silencing [3], cell proliferation [2,4] and DNA repair or apoptosis [3]. Third, biotin affects gene expression at both the transcriptional [5–7] and the posttranscriptional level [6,8,9]. Numerous cell signals have been identified that mediate effects of biotin on gene expression: biotinyl-AMP [10], transcription factors such as NF-κB [11], Sp1 and Sp3 [12], and biotinylation of histones [13].

Abbreviations: ATF6, activating transcription factor 6; eIF2α, eukaryotic translation initiation factor 2α; EMSA, electrophoretic mobility shift assay; *GADD153*, *growth arrest and DNA damage 153* gene; Grp78, glucose regulated protein 78; IL-2, interleukin-2; PCC, propionyl-CoA carboxylase; PHA, phytohemagglutinin; PMA, phorbol-12-myristate-13-acetate; RT-PCR, reverse transcriptase–polymerase chain reaction; SERCA3, sarco/endoplasmic reticulum Ca^{2+}-ATPase 3; UBE1, ubiquitin-activating enzyme 1; UPR, unfolded protein response; XBP1, X-box binding protein 1.

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Approximately 270 biotin-dependent genes have been identified in human lymphocytes by using DNA microarrays [14]. In these previous studies, gene expression profiles were obtained before and after supplementation of healthy adults with 8.8 μmol of biotin per day for 21 days. Sarco/endoplasmic reticulum Ca^{2+}-ATPase 3 (SERCA3) was among the genes that were down-regulated substantially in response to biotin supplementation: the abundance of mRNA encoding SERCA3 (isoform a) decreased by >80% in post-supplementation compared to pre-supplementation lymphocytes; expression of isoforms b, c, d and e was quantitatively negligible in lymphocytes. The 5′-flanking region of the *SERCA3* gene contains 25 consensus binding sites for the biotin-dependent transcription factors Sp1 and Sp3 [12,15]. Sp1 and Sp3 may act as transcriptional activators or repressors, depending on the context [16,17]. Based on these data, it seems likely that the decreased expression of SERCA3 in biotin-supplemented cells is mediated by increased abundance of Sp1 and Sp3.

Three homologous genes encode for the calcium transporters SERCA1, SERCA2 and SERCA3 [18,19]. These proteins transport calcium from the cytoplasm into the endoplasmic reticulum (ER), maintaining a resting concentration of free Ca^{2+} in the ER that is three to four orders of magnitude greater than in the cytoplasm [19]. SERCA3 is abundantly expressed in lymphoid cells [20], suggesting a pivotal role for this transporter in the calcium homeostasis in immune cells.

Decreased expression of SERCA3 in response to biotin supplementation is likely to affect the oxidative folding (formation of disulfide bonds) of secretory proteins in the ER, based on the following reasons. Secretory proteins enter the ER co-translationally for glycosylation and oxidative folding by calcium-dependent proteins such as calnexin [21,22], BiP [23], and protein disulfide isomerase [23]. Glycosylation and folding in the ER are essential for subsequent protein export [24,25]. Depletion of ER-resident Ca^{2+} by ionophores, chelators or inhibitors of SERCA results in accumulation of immature proteins in the ER [25–29]. Accumulation of immature proteins triggers stress-response systems collectively referred to as unfolded protein response (UPR) [30]. UPR is characterized by high proteolytic activity [31], increased expression of chaperones that mediate protein folding [32,33], low overall translational activity [30] and transcriptional activation of *SERCA* genes [34]. Continued accumulation of unfolded proteins causes growth arrest and apoptosis [35].

In the present study we tested the hypothesis that supplementation of human lymphoma (Jurkat) cells with pharmacological doses of biotin impairs the posttranslational processing of secretory proteins, causing cell stress. Specifically, we determined (i) whether biotin supplementation decreases the expression of SERCA3, impairing the sequestration of Ca^{2+} in the ER; (ii) whether the biotin-dependent disturbance of calcium homeostasis is associated with decreased secretion of proteins into the extracellular space; and (iii) whether biotin supplementation activates the UPR.

Jurkat cells were selected as a cell model for the following reasons. First, biotin-dependent signaling cascades have been characterized in Jurkat cells [11,12]. Second, biotin-dependent repression of the *SERCA3* gene [14] is likely to affect Ca^{2+} homeostasis in lymphoma cells [20]. Third, lymphoid cells such as Jurkat cells secrete proteins that play essential roles in immune function [36]. Fourth, Jurkat cells have been used successfully in previous studies to investigate effects of nutrients on UPR [37].

2. Materials and methods

2.1. Cell culture

Jurkat cells (clone E6-1) were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in the following biotin-defined media for at least 5 weeks prior to sample collection [38]: (i) 0.025 nmol/L of biotin (denoted "deficient"), representing the plasma level in biotin-deficient individuals; or (ii) 10 nmol/L of biotin ("supplemented"), representing the plasma level in individuals who take typical over-the-counter supplements providing 20 times the adequate intake of biotin [39–41]. Culturing Jurkat cells in biotin-defined media for 5 weeks provides for sufficient time to achieve new steady-state concentrations of biotin, as judged by activities of PCC and biotinylation of carboxylases [38]. Culture media were replaced with fresh media every 48 h. Media were prepared by using customized RPMI-1640 and biotin-depleted fetal bovine serum as described [38]; biotin concentrations in media were confirmed by avidin-binding assay [42] with modifications [38]. For some control experiments, we cultured human HepG2 hepatocarcinoma cells in biotin-defined media as described [43].

2.2. Biotin-dependent carboxylases

Biotin-dependent carboxylases are good markers for cellular biotin [1]. Biotinylated carboxylases in cell extracts were resolved by polyacrylamide gel electrophoresis and were probed using streptavidin peroxidase [38]. Densitometric analysis of gels was conducted as described [12]. Activities of PCC in cell lysates were quantified as described [38].

2.3. Reporter gene constructs

The following reporter gene constructs were used to quantify effects of biotin on the transcriptional activity of the *SERCA3* gene [15]: (i) −1312 to +55 bp (+1=transcription start site) from the 5′-flanking region of the human *SERCA3* gene (*ATP2A3*) driving luciferase expression (denoted "*Eco*RV-del."); (ii) −1043 to +55 bp of *ATP2A3* driving luciferase expression ("*Hin*dIII-del."); (iii) −731 to +55 bp of *ATP2A3* driving luciferase expression ("*Sac*I-del."); (iv) −351 to +55 bp of *ATP2A3* driving luciferase

expression ("*Eco*RI-del."); (v) −135 to +55 bp of *ATP2A3* driving luciferase expression ("*Pst*I-del."); and (vi) −31 to +55 bp of *ATP2A3* driving luciferase expression ("*Sma*I-del."). A promoter-free plasmid containing the luciferase gene (denoted "pGL3-Basic") was purchased from Promega (Madison, WI); a construct of the β-galactosidase reporter gene driven by the RSV promoter (denoted "RSV βgal") was obtained from B.R. White (University of Nebraska-Lincoln). Transfections and reporter gene analyses were conducted as described, including stimulation with phorbol-12-myristate-13-acetate (PMA) and phytohemagglutinin (PHA) [44]. Reporter gene activities were quantified 48 h after transfection. Luciferase activities were normalized for transfection efficiency by using β-galactosidase activities. Data are expressed as ratios of luciferase activities in cells transfected with promoter-driven constructs to activities in cells transfected with pGL3-Basic.

Accumulation of unfolded proteins in the ER causes nuclear translocation of activating transcription factor 6 (ATF6) [45] and X-box binding protein 1 (XBP1) [32]; these transcription factors bind to ER stress elements (ERSE) in 5′-flanking regions of stress-response genes [32,45,46]. The following reporter gene constructs were used to determine whether transcriptional activities in biotin-supplemented cells are consistent with UPR mediated by ERSE. (i) A reporter construct in which expression of luciferase is driven by five repeats of a consensus binding site for ATF6 [47]: "p5xATF6-GL3." This plasmid was provided R. Prywes (Columbia University, NY) [47]. (ii) Reporter constructs in which expression of luciferase is driven by 169 and 457 bp, respectively, upstream from the transcription start site in the *glucose regulated protein 78* (*Grp78*) gene: "–169GRP78-luc" and "–457GRP78-luc" [48]. These plasmids were provided by A.S. Lee (University of Southern California, Los Angeles, CA). The GRP78/BiP protein binds misfolded proteins in the ER, mediating their refolding or degradation [33]. (iii) A reporter construct in which expression of luciferase is driven by the ERSE from the human *growth arrest and DNA damage 153* (*GADD153*) gene: "CHOP-ERSE1"; baseline luciferase activity was quantified by using plasmid "CHOP-ERSE1(mut8)" which is not inducible by ER stress [49]. Both CHOP-ERSE1 and CHOP-ERSE1(mut8) were provided by C.C. Glembotski (San Diego University, CA) [49]. GADD153 is a pro-apoptotic transcription factor (synonymous to CHOP); abundance of GADD153 increases in response to accumulation of unfolded proteins [30]. Transfections and reporter gene analyses were conducted as described above.

2.4. Northern blots

Expression of SERCA3 and histone H4 (control) was quantified by Northern blot analysis as described [50]. Gene-specific probes for Northern blots were generated by polymerase chain reaction as described [50] using the following primers: (i) 5′-GAG TCA CGC TTC CCC ACC ACC-3′ and 5′-GGC TCA TTT CTT CGT GCA TGT GGT TC-3′ for human SERCA3 (GenBank accession number NM_005173); and (ii) 5′-ATG TCT GGT AGA GGC AAA GGT GGT AAA-3′ and 5′-TCA GCC ACC AAA GCC GTA CAG AGT GCG-3′ for histone H4 (GenBank accession number M60749). Identities of probes were confirmed by sequencing in the Genomic Research Core Facility at the University of Nebraska-Lincoln (data not shown). Abundance of mRNA was quantified by gel densitometry [50]; the abundance of SERCA3 mRNA was normalized by the abundance of histone H4 mRNA.

2.5. Cytoplasmic Ca^{2+}

Incubation of lymphoid cells with PMA and PHA causes release of Ca^{2+} from intracellular stores into the cytoplasm [36]. Here, cytoplasmic free Ca^{2+} was quantified at timed intervals before and after treatment of cells with 50 μg/L of PMA and 2 mg/L of PHA; Ca^{2+} was quantified using the Fluo-3 AM kit (Molecular Probes, Eugene, OR) in a 96-well plate format according to the manufacturer's instructions. In addition, cytoplasmic Ca^{2+} was visualized by using confocal microscopy and the Fluo-3 AM kit (Microscopy Core Facility at the University of Nebraska-Lincoln).

2.6. Secretion of interleukin-2

Jurkat cells secrete interleukin-2 (IL-2) in response to appropriate stimulation; IL-2 contains one disulfide bond and, thus, requires oxidative folding prior to secretion [37]. Here, cells were stimulated with PMA and PHA to induce secretion of IL-2 [37]. IL-2 in culture media was quantified using ELISA ("hIL-2 EASIA," Biosource, Camarillo, CA) as described previously [38].

2.7. Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were used to determine whether biotin supplementation affects the nuclear abundance of transcription factors with affinity for ERSE. Nuclear extracts from Jurkat cells were prepared as described previously [12]. ERSE-binding factors in nuclear extracts were probed by EMSA [11] using a ^{32}P-labeled double-stranded oligonucleotide probe with an ERSE consensus site for ATF6 and XBP1: 5′-GAG GGC CTT CAC CAA TCG GCG GCC TCC ACG ACG GGG CTG G-3′ and 5′-CCA GCC CCG TCG TGG AGG CCG CCG ATT GGT GAA GGC CCT C-3′ [32]. The nuclear abundance of the transcription factor Oct-1 does not depend on biotin and was quantified as a control [12]. Additional controls were prepared by omitting nuclear extracts from incubation mixtures, by incubating nuclear extracts with radiolabeled probe in the presence of a molar excess of unlabeled probe and by using nuclear extracts from HepG2 cells. Binding of transcription factors to ERSE was quantified by using gel densitometry [12].

In some cases, transcription factor–oligonucleotide complexes were supershifted by using polyclonal rabbit IgG antihuman antibodies to XBP1 (Santa Cruz Biotechnology,

Santa Cruz, CA). These samples were prepared by incubating 5 μg of nuclear protein with 2 μg of antibody to XBP1 for 60 min at 4°C prior to adding oligonucleotide probes. Note that incubation of nuclear proteins with antibodies (i) may prevent formation of transcription factor–oligonucleotide complexes (if antibodies block oligonucleotide-binding sites of transcription factors); or (ii) may decrease the electrophoretic mobility of the transcription factor–oligonucleotide complex (if antibodies bind to transcription factors without blocking their oligonucleotide-binding sites).

2.8. Reverse transcriptase–polymerase chain reaction

Expression of genes encoding XBP1 and β-actin (control) was quantified by reverse transcriptase–polymerase chain reaction (RT-PCR) as described [51]. The following customized primers were used for PCR (Integrated DNA Technologies, Coralville, IA): (i) 5′-TGG TAG ATT TAG AAG AAG AGA ACC AAA-3′ and 5′-AAT CAG CTG GGG AAA GAG TTC ATT GGC-3′ for XBP1 (GenBank accession number AB_76384); and (ii) 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′ and 5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′ for β-actin (GenBank accession number NM_001101). cDNA was quantified by gel densitometry using the Kodak EDAS 290 Documentation and Analysis System (Rochester, NY); only values from within the exponential phase of PCR amplification were considered for data analysis. Abundance of cDNA encoding XBP1 was normalized by the abundance of cDNA encoding β-actin.

2.9. Western blot analyses

The following proteins play a role in the cellular response to ER stress: (i) the transcription factor XBP1 (see above); (ii) the pro-apoptotic transcription factor GADD153 (see above); (iii) ubiquitin-activating enzyme 1 (UBE1): UBE1 catalyzes the first step in the proteasome-dependent degradation of unfolded proteins [52,53]; accumulation of unfolded proteins is associated with increased expression of UBE1 [37]; and (iv) eukaryotic translation initiation factor 2α (eIF2α): eIF2α is part of the active 40 S translation preinitiation complex [54]. Cells respond to ER stress with phosphorylation of a serine residue in eIF2α; phosphorylated eIF2α cannot participate in the formation of the preinitiation complex [54], decreasing both translational activity and the abundance of unfolded proteins in the ER [26,28].

For analysis of GADD153 and UBE1, protein extracts were prepared from whole cells by using detergents and protease inhibitors [55]. Nuclear extracts for analysis of XBP1 were prepared by using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. For analysis of phosphorylated eIF2α, cytoplasmic protein extracts were prepared in the presence of phosphatase inhibitors [56]. Nuclear histone H3 (control) was prepared by acid extraction as described [2]. The following gels were used to resolve proteins (Invitrogen, Carlsbad, CA) as described previously [2,12,55]: 18% Tris–glycine gels (GADD153, UBE1 and histone H3) and 4–12% Bis–Tris gels (XBP1 and eIF2α). Proteins were probed with the following antibodies using standard Western blot procedures [55]: rabbit polyclonal antihuman XBP1 (Santa Cruz Biotechnology), mouse monoclonal antihuman GADD153 (Santa Cruz Biotechnology), mouse monoclonal antihuman UBE1 (Upstate Biotechnologies, Lake Placid, NY), rabbit polyclonal antihuman phospho-eIF2α (Upstate Biotechnologies) and goat polyclonal antihuman histone H3 (Santa Cruz Biotechnology).

2.10. Statistical analysis

The paired t test was used to test for significance of differences [57]. StatView 5.0.1 (SAS Institute, Cary, NC) was used to perform all calculations. Differences were considered significant if $P<.05$. Data are expressed as mean ± S.D.

3. Results

3.1. Biotin-dependent carboxylases

Biotinylation of carboxylases in Jurkat cells paralleled biotin concentrations in culture media. If cells were cultured in biotin-deficient medium, biotinylated pyruvate carboxylase, PCC and 3-methylcrotonyl-CoA carboxylase were barely detectable by Western blot analysis of cell extracts using streptavidin as a probe (Fig. 1A). In contrast, holocarboxylases were abundant in extracts from cells cultured in biotin-supplemented medium. Note that the biotin-containing α chains of PCC (molecular mass=80 kDa) and 3-methylcrotonyl-CoA carboxylase (molecular mass=83 kDa) migrate as one single band on the gels used here. Holocarboxylases were 49–78 times more abundant in biotin-supplemented cells than in biotin-deficient cells, as judged by gel densitometric analysis of Western blots (Fig. 1B). Acetyl-CoA carboxylase was not detectable in cell extracts, consistent with previous studies in Jurkat cells [38].

PCC activities paralleled the abundance of holocarboxylases in Jurkat cells. PCC activity was 60±5.1 pmol bicarbonate fixed/(min×10^6 cells) and 24±2.8 pmol bicarbonate fixed/(min×10^6 cells) in biotin-supplemented and biotin-deficient cells, respectively ($P<.01$; $n=5$). Collectively, these findings suggest that biotin concentrations in culture media affected intracellular biotin concentrations.

3.2. SERCA3 expression

Biotin concentrations in culture media affected the transcriptional activity of the SERCA3 promoter. The transcriptional activity of the full-length construct (−1312 to +55 bp) was 50% smaller in biotin-supplemented cells

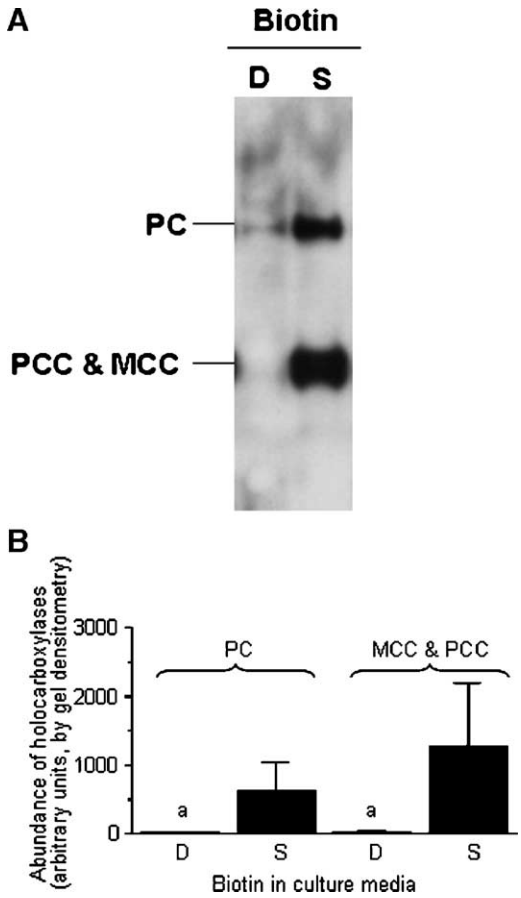

Fig. 1. Biotin supply affects the abundance of holocarboxylases in Jurkat cells. Cells were cultured in biotin-deficient ("D"; 0.025 nmol/L) and biotin-supplemented ("S"; 10 nmol/L) media for 5 weeks. (Panel A) Representative Western blot depicting pyruvate carboxylase (PC), α-chain of PCC, and α-chain of 3-methylcrotonyl-CoA carboxylase (MCC). (Panel B) Gel densitometric analysis of Western blots. Values are means±S.D. ($n=6$). [a]$P<.05$, significantly different from the abundance of the same carboxylase in cells cultured in biotin-supplemented medium.

compared to biotin-deficient cells (Fig. 2). Effects of biotin on transcriptional activity decreased if −1312 to −731 bp were deleted, consistent with the binding of biotin-dependent repressors to this region. This region contains three Sp1 sites: one Ets-1 site, one GATA box and one Oct-1 binding site [15]. Further deletion of −731 to −135 bp reversed the effects of biotin on transcriptional activity: biotin supplementation was associated with a moderate transcriptional activation of *SERCA3*. Likely, the region spanning −731 to −135 bp contains binding sites for biotin-dependent activators of transcription; this region contains nine Sp1 sites [15]. Note that plasmid *Eco*RV-del (−1312/+55) is the full-length construct and, thus, is most likely to represent effects of biotin on transcriptional activity of the *SERCA3* gene in vivo.

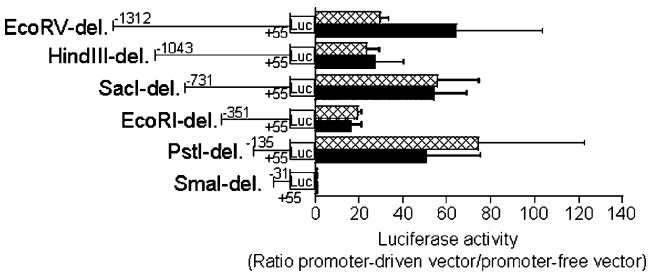

Fig. 2. The transcriptional activity of the human SERCA3 promoter depends on biotin. Biotin-supplemented (hatched bars) and biotin-deficient (solid bars) Jurkat cells were transfected with reporter (luciferase) plasmids driven by the full-length or truncated forms of the 5′ -flanking region of SERCA3. Forty-eight hours after transfection, cells were stimulated with PMA and PHA for 6 h. Data are expressed as ratios of luciferase activities in cells transfected with SERCA3 constructs to activities in cells transfected with promoter-free vector ($n=5–6$).

Next, the abundance of SERCA3 mRNA in Jurkat cells was quantified by Northern blot analysis to confirm biotin-dependent transcription of the *SERCA3* gene in human lymphoid cells. The abundance of SERCA3 mRNA was approximately 50% smaller in biotin-supplemented cells compared to biotin-deficient cells (Fig. 3A and B). Collectively, previous studies in human lymphocytes [14] and the present studies in Jurkat cells are consistent with decreased expression of the *SERCA3* gene in biotin-supplemented compared to biotin-deficient lymphoid cells.

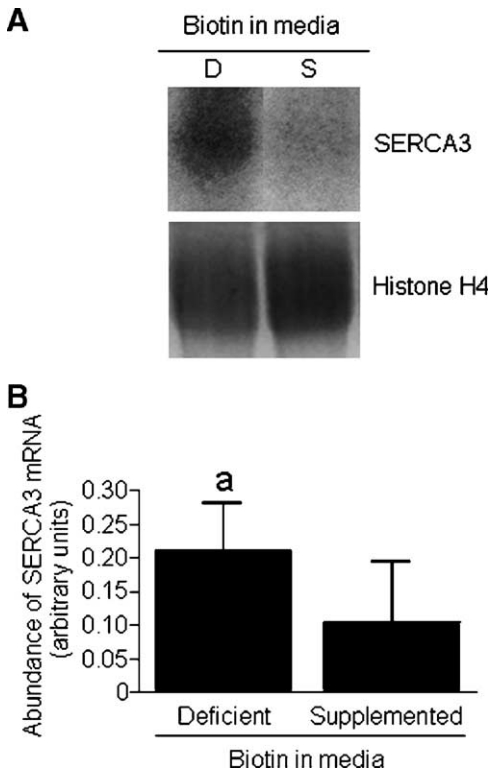

Fig. 3. Biotin affects the abundance of SERCA3 mRNA in Jurkat cells. Cells were cultured in biotin-deficient ("D"=0.025 nmol/L) and biotin-supplemented ("S"=10 nmol/L) media for 5 weeks. (Panel A) Representative Northern blots of SERCA3 and histone H4 (control). (Panel B) Gel densitometric quantitation of SERCA3 mRNA, normalized by the abundance of histone H4 mRNA. Values are means±S.D. ($n=3$). [a]$P<.05$, significantly different from cells cultured in biotin-supplemented medium.

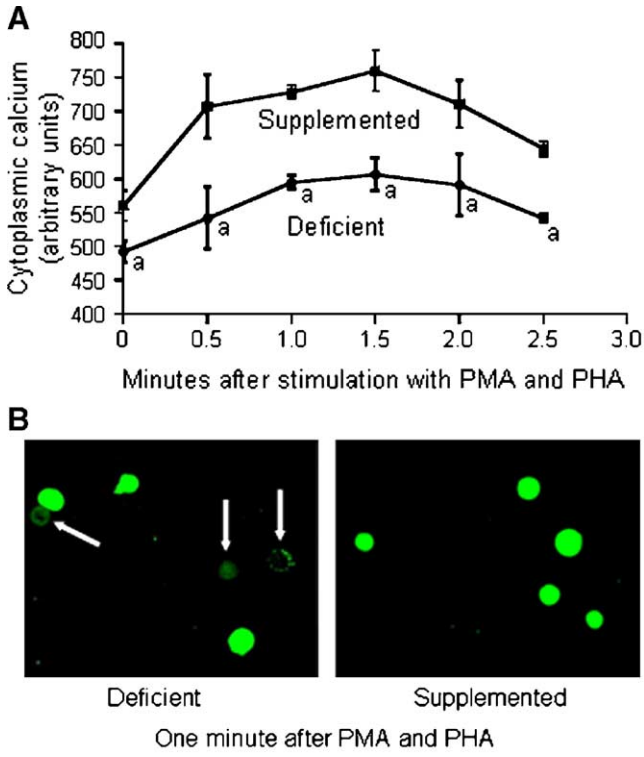

Fig. 4. Biotin affects the concentration of free calcium in Jurkat cell cytoplasm. Cells were cultured in biotin-deficient (0.025 nmol/L biotin) and biotin-supplemented (10 nmol/L biotin) media for 5 weeks. (Panel A) Cells were stimulated with PMA and PHA, and cytoplasmic free calcium was probed at timed intervals using a fluorophore. Values are means ± S.D. ($n=5$). [a]$P<.01$, significantly different from cells cultured in biotin-supplemented medium. (Panel B) Images were obtained by confocal microscopy 1 min after stimulation of cells with PMA and PHA. Arrows depict cells with low cytoplasmic calcium.

3.3. Cytoplasmic Ca^{2+}

The concentration of free Ca^{2+} in the cytoplasm depended on biotin. The cytoplasmic Ca^{2+} concentration was approximately 14% greater in resting biotin-supplemented cells compared to resting biotin-deficient cells (Fig. 4). Cytoplasmic concentrations of Ca^{2+} increased if cells were stimulated with PMA and PHA, consistent with the release of Ca^{2+} from intracellular stores. The cytoplasmic concentration of Ca^{2+} was approximately 24% greater in biotin-supplemented compared to biotin-deficient cells after stimulation with PMA and PHA. These observations are consistent with decreased expression of the SERCA3 pump in biotin-supplemented cells, reducing the transport of Ca^{2+} from the cytoplasm into the ER.

3.4. Secretion of IL-2

Secretion of IL-2 requires oxidative folding in the ER, leading to the formation of a disulfide bond between cysteine-58 and cysteine-105 [58]. In the present study, secretion of IL-2 was smaller in biotin-supplemented compared to biotin-deficient cells [kU of IL-2/(L×10^6 cells×6 h)]: 3.4±0.3 vs. 13.6±0.3 ($n=7$; $P<.01$). This finding suggests that the oxidative folding of secretory proteins is impaired in biotin-supplemented cells.

3.5. Electrophoretic mobility shift assay

ERSE-binding transcription factors were detected in nuclear extracts from both biotin-deficient and biotin-supplemented Jurkat cells, but the DNA-binding activity of these transcription factors was not affected by biotin (Fig. 5, lanes 1 and 2). The following controls suggested that the observed effects were specific. First, binding of transcription factors to ERSE was eliminated by pretreating nuclear extracts with an antibody to XBP1 (lanes 3 and 4). Second, no bands were detectable in the absence of nuclear extract (lane 5) or in the presence of a 50-fold molar excess of unlabeled probe (lane 6). Finally, biotin concentrations in culture media did not affect the nuclear abundance of Oct-1 (Fig. 5, lanes 7 and 8).

Previous studies suggested that Jurkat cells are less sensitive than HepG2 cells to diet-induced ER stress [37,59]. Hence, we tested HepG2 cells to determine whether biotin supplementation is associated with increased nuclear ERSE-binding activity in biotin-supplemented cells. Indeed, ERSE-binding activity was greater in biotin-supplemented HepG2 cells compared with biotin-deficient cells (Fig. 5, lanes 9 and 10). This observation was corroborated by using gel densitometric analysis of EMSA. ERSE-binding activity equaled 238±19 arbitrary units in biotin-supplemented HepG2 cells compared with 155±32 arbitrary units in biotin-deficient cells ($P<.05$; $n=3$).

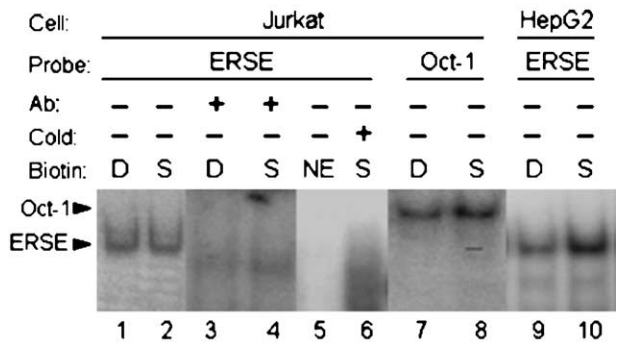

Fig. 5. Nuclear translocation of ERSE-binding factors increases in response to biotin supplementation in HepG2 cells but not in Jurkat cells. Cells were cultured in biotin-deficient ("D"=0.025 nmol/L) and biotin-supplemented ("S"=10 nmol/L) media for 10 days (HepG2 cells) and 5 weeks (Jurkat cells), respectively. Nuclear extracts were analyzed by EMSA for DNA-binding activities of transcription factors: lanes 1 and 2, ERSE-binding activity in Jurkat nuclear extracts; lanes 3 and 4, Jurkat nuclear were pretreated with anti-XBP1 antibody; lane 5, absence of nuclear extract; lane 6, ERSE-binding activity in Jurkat nuclear extracts in the presence of a 50-fold molar excess of unlabeled ERSE; lanes 7 and 8, DNA-binding activity of Oct-1 in Jurkat nuclear extracts; lanes 9 and 10, ERSE-binding activity in HepG2 nuclear extracts. NE, no nuclear extract; Ab, antibody-treated (supershift experiment).

3.6. Transcriptional activities of ERSE-dependent reporter genes

Jurkat cells responded to supplementation with pharmacological doses of biotin by increased transcriptional activities of the following ERSE-dependent reporter genes. (i) The transcriptional activity (luciferase activity) of a reporter gene (p5xATF6-GL3) driven by five ERSE was 22% greater in biotin-supplemented compared to biotin-deficient cells (Table 1). (ii) The transcriptional activities (luciferase activity) of reporter genes driven by the 5′-flanking region of the *Grp78* gene were 271% (plasmid –169GRP78-luc) and 196% (plasmid –457GRP78-luc) greater in biotin-supplemented compared to biotin-deficient cells (Table 1). Collectively, these findings are consistent with ER stress caused by biotin supplementation. (iii) The transcriptional activity (luciferase activity) of a reporter gene (CHOP-ERSE1) driven by the ERSE in the *GADD153* gene was 53% greater in biotin-supplemented compared to biotin-deficient cells (Table 1).

3.7. Abundance of stress response proteins

The cellular abundance of the following stress-related proteins was greater in biotin-supplemented compared to biotin-deficient cells (Fig. 6): UBE1 in whole cell extracts, GADD153 in whole cell extracts, XBP1 in nuclear extracts and phosphorylated eIF2α in cytoplasmic extracts. The abundance of a control protein (nuclear histone H3) was not affected by biotin. These findings are consistent with ER stress caused by biotin supplementation.

3.8. XBP1 expression

The abundance of mRNA encoding XBP1 was moderately greater in biotin-supplemented compared to biotin-deficient cells, as judged by RT-PCR and gel densitometric analysis (arbitrary units): 478±118 vs. 438±152 (n=3). However, the difference was not statistically significant (P=.20). The abundance of β-actin mRNA (control) was not affected by biotin. The increased nuclear abundance of XBP1 protein observed in biotin-supplemented cells might be caused by mechanisms other than increased transcription of the XBP1 gene.

Table 1
Effects of biotin concentrations in culture media on the transcriptional activities of ERSE-dependent luciferase constructs in Jurkat cells

Plasmid	Biotin in culture medium (nmol/L)	
	0.025	10
Stimulation of luciferase activity[a]		
p5xATF6-GL3	2.6±0.22*	3.2±0.04
–169GRP78-luc	49±3.5**	182±15.4
–457GRP78-luc	114±21**	338±68
CHOP-ERSE1	1.5±0.07**	2.3±0.02

[a] Values are ratios of luciferase activities in cells transfected with ERSE-dependent luciferase constructs to activities in cells transfected with promoter-free luciferase constructs.

* P=.05, significantly different compared to cells cultured in medium containing 10 nmol/L biotin (n=3 independent experiments).

** P<.01, significantly different compared to cells cultured in medium containing 10 nmol/L biotin (n=3 independent experiments).

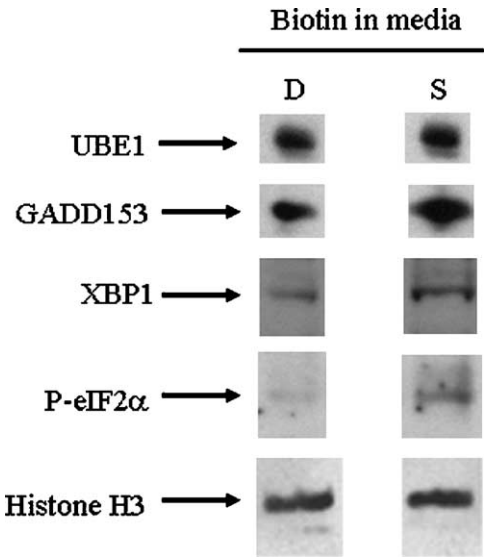

Fig. 6. Abundance of ER stress-related proteins depends on biotin in Jurkat cells. Cells were cultured in biotin-deficient ("D"=0.025 nmol/L) and biotin-supplemented ("S"=10 nmol/L) media for 5 weeks. The following proteins were probed by Western blot analysis: UBE1 in whole cell extracts, GADD153 in whole cell extracts, XBP1 in nuclear extracts and phosphorylated eIF2α (P-eIF2α) in cytoplasmic extracts. Histone H3 in nuclear extracts was used as a control.

4. Discussion

The present study provides evidence that biotin supplementation is associated (i) with decreased expression of the *SERCA3* gene in human lymphoid cells; (ii) with decreased sequestration of calcium in the ER; (iii) with decreased secretion of IL-2 into the extracellular space; (iv) with increased nuclear abundance of transcription factors binding to ERSE; (v) with increased transcriptional activity of ERSE-dependent genes; and (vi) with increased cellular abundance of proteins that play roles in UPR. Collectively, these findings are consistent with the hypothesis that supplementation with pharmacological doses of biotin may cause cell stress by impairing protein folding in the ER.

Effects of biotin on calcium flux and protein folding are physiologically important, based on the following reasons. First, some cell types depend on rapid proliferation in order to maintain normal physiological responses, for example, antibody-secreting cells [36]. Cell stress triggering UPR is associated with decreased cell proliferation and increased cell death [30]. Thus, biotin-induced UPR might decrease the number of secretory cells, affecting processes such as the immune response. Second, UPR is associated with decreased secretion of disulfide-containing proteins into the extracellular space [29]. Secretory proteins such as IL-2 play essential roles in various physiological processes. For

example, extracellular IL-2 binds to receptors located on the surface of target cells such as T and B cells and natural killer cells, triggering proliferation and differentiation [36]. Decreased abundance of IL-2 is likely to impair immune function. Third, cytoplasmic free Ca^{2+} enhances the generation of the second messenger nitric oxide in Jurkat cells [60]. Ongoing studies in our laboratory suggested that biotin supplementation is associated with increased synthesis of nitric oxide (R. Rodriguez-Melendez and J. Zempleni, unpublished observation); biological effects of biotin-induced generation of nitric oxide are currently being investigated.

Classically, the use of dietary supplements containing pharmacological doses of biotin has been considered safe [1]. The findings presented here suggest that a careful reevaluation of the safety of biotin supplementation might be in order. Biotin supplementation may be associated with an undesired pattern of gene expression such as decreased expression of SERCA3 (present study) and increased expression of cytochrome *P*450 1B1 [61]. Cytochrome *P*450 1B1 catalyzes hydroxylation of procarcinogens such as polycyclic aromatic hydrocarbons, aryl amines [62] and 17β-estradiol [63]. This metabolic activation of procarcinogens is a key factor in carcinogenesis of the human prostate [64,65], breast [66] and uterus [67]. Collectively, effects of biotin on gene expression [5,14] and cell signaling [11,12] need to be considered when establishing tolerable upper intake levels for biotin [40].

The present study suggests that biotin-dependent repressors bind to elements located 731–1312 bp upstream from the transcription start site in the *SERCA3* gene. The identity of these repressors is unknown. The following response elements have been identified in the region spanned by −731 to −1312 bp: three Sp1 sites, one Ets-1 site, one GATA box and one Oct-1 binding site [15]. The present study and previous studies [11,12] provided evidence that biotin does not affect the nuclear abundance of Oct-1, suggesting that effects of biotin on SERCA3 expression are not mediated by Oct-1. In contrast, the nuclear abundance of Sp1 and Sp3 correlates positively with biotin supply [12]. Given that Sp3 may act as a transcriptional repressor [16,17,68], Sp3 might mediate the decreased expression of SERCA3 in biotin-supplemented cells. However, this is an untested speculation, and unidentified proteins and signaling pathways might also play a role in biotin-dependent repression of SERCA3.

The present study provides evidence that biotin supplementation might impair protein folding, decreasing the secretion of proteins such as IL-2 into the extracellular space. Note that factors other than protein folding may also affect the extracellular concentration of IL-2. Previously we have demonstrated that expression of IL-2 receptor γ correlates positively with biotin supply in Jurkat cells [44]. Increased expression of IL-2 receptor γ is associated with increased endocytosis of IL-2, decreasing the concentration of IL-2 in the culture medium.

Notwithstanding the above-mentioned uncertainties, the present study is consistent with the hypothesis that biotin overdose may interfere with Ca^{2+} homeostasis in Jurkat cells. Jurkat cells fold and secrete relatively moderate amounts of proteins, that is, <100 pg of IL-2/(10^6 cells×h) [44]. It will be of interest to determine whether cells that secrete larger amounts of proteins (e.g., antibody-producing cells) are more susceptible to triggering UPR in response to biotin supplementation.

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