

Riboflavin deficiency causes protein and DNA damage in HepG2 cells, triggering arrest in G1 phase of the cell cycle[☆]

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Abstract

Eukaryotes convert riboflavin to flavin adenine dinucleotide, which serves as a coenzyme for glutathione reductase and other enzymes. Glutathione reductase mediates the regeneration of reduced glutathione, which plays an important role in scavenging free radicals and reactive oxygen species. Here we tested the hypothesis that riboflavin deficiency decreases glutathione reductase activity in HepG2 liver cells, causing oxidative damage to proteins and DNA, and cell cycle arrest. As a secondary goal, we determined whether riboflavin deficiency is associated with gene expression patterns indicating cell stress. Cells were cultured in riboflavin-deficient and riboflavin-supplemented media for 4 days. Activity of glutathione reductase was not detectable in cells cultured in riboflavin-deficient medium. Riboflavin deficiency was associated with an increase in the abundance of damaged (carbonylated) proteins and with increased incidence of DNA strand breaks. Damage to proteins and DNA was paralleled by increased abundance of the stress-related transcription factor GADD153. Riboflavin-deficient cells arrested in G1 phase of the cell cycle. Moreover, oxidative stress caused by riboflavin deficiency was associated with increased expression of clusters of genes that play roles in cell stress and apoptosis. For example, the abundance of the pro-apoptotic *pleiomorphic adenoma gene-like 1* gene was 183% greater in riboflavin-deficient cells compared with riboflavin-sufficient controls. We conclude that riboflavin deficiency is associated with oxidative damage to proteins and DNA in liver cells, leading to cell stress and G1 phase arrest.

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1. Introduction

Riboflavin is a precursor of flavin mononucleotide and flavin adenine dinucleotide (FAD), which serve as coenzymes for numerous oxidases and dehydrogenases in eukaryotic cells [1]. For example, FAD-dependent Ero1 and sulfhydryl oxidases participate in the oxidative folding (formation of disulfide bonds) of secretory proteins in the endoplasmic reticulum [2–5]. Riboflavin deficiency impairs

the oxidative folding and subsequent secretion of proteins in human cell cultures [4,5]. Consequently, unfolded proteins accumulate in the endoplasmic reticulum of riboflavin-deficient cells [4,5], triggering a stress response named the *unfolded protein response* [6]. This response may include (i) phosphorylation of the eukaryotic initiation factor 2 α , attenuating global translational activity [5,7]; (ii) expression of chaperones assisting in protein folding [5,7]; and (iii) activation of ubiquitin-dependent degradation of misfolded proteins [5,8]. Importantly, accumulation of unfolded proteins is associated with decreased proliferation rates in riboflavin-deficient cells [5]; activation of the transcription factor GADD153 appears to play a role in this process [5,9]. Finally, some evidence for increased apoptotic activity was observed in riboflavin-deficient cells [5].

Theoretically, effects of riboflavin deficiency on cell proliferation and apoptosis may not be specific for the unfolded protein response, but may also be caused by

Abbreviations: FAD, flavin adenine dinucleotide; RT-PCR, reverse transcriptase–polymerase chain reaction.

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decreased activities of other flavin-dependent pathways. In particular, FAD serves as a coenzyme for glutathione reductase, an enzyme essential for regenerating reduced glutathione [1]. Riboflavin deficiency is associated with decreased activities of glutathione reductase and with decreased concentrations of reduced glutathione in human cells [4,5]. Reduced glutathione scavenges free radicals and other reactive oxygen species, preventing oxidative damage to proteins, DNA and other compounds [10,11]. Moreover, *S*-glutathionylation of sulfhydryl groups in cysteines depends on reduced glutathione; *S*-glutathionylation plays essential roles in the regulation of protein function [12]. Accumulation of oxidative damage in proteins and DNA may trigger stress responses such as nuclear translocation of nuclear factor κ B, cell cycle arrest and apoptosis [13,14]. Here we tested the hypothesis that some of the known effects of riboflavin deficiency on cell proliferation and apoptosis are caused by damage to proteins and DNA. Specifically, we determined whether riboflavin deficiency (i) causes accumulation of carbonyl groups in amino acid residues of proteins, (ii) increases the frequency of DNA strand breaks, (iii) causes cell cycle arrest and (iv) mediates pro-apoptotic and stress-related gene expression patterns in human cells. Hepatocarcinoma (HepG2) cells are prone to developing riboflavin deficiency [5] and were used as models in these studies.

2. Materials and methods

2.1. Cell culture

HepG2 cells were purchased from ATCC (Manassas, VA). For cell stress experiments, HepG2 cells were cultured in customized RPMI-1640 containing the following concentrations of riboflavin for 4 days: 3.1 nmol/L (denoted “deficient”) and 300 nmol/L (“supplemented”) [5]. Media contained 10% dialyzed, riboflavin-depleted bovine growth serum [4]. Riboflavin concentrations in media were chosen based on the following. First, previous studies provided evidence that HepG2 cells develop signs of riboflavin deficiency if cultured in medium containing 3.1 nmol/L riboflavin [5]; this concentration represents the level of riboflavin observed in plasma from moderately deficient pregnant women [15]. Second, previous studies provided evidence that a concentration of 300 nmol/L riboflavin in culture medium prevents signs of riboflavin deficiency in HepG2 cells [5]; this concentration represents the level of riboflavin observed in plasma from individuals taking riboflavin supplements [16] and in portal vein blood [5,17]. The portal vein provides the liver with water-soluble nutrients obtained through intestinal absorption. For DNA microarray experiments we included a third treatment group: cells cultured in medium containing 12.6 nmol/L riboflavin. This represents the riboflavin concentration in normal human plasma [16] and was designated the control group in microarray experiments.

2.2. Glutathione metabolism

The activity of glutathione reductase was quantified in lysed HepG2 cells as described previously [18] with minor modifications [4].

2.3. Western blot analyses

Here we quantified carbonylated proteins and the transcription factor GADD153. Carbonylation of proteins is a marker for oxidative protein damage [19]; GADD153 mediates cell growth arrest and apoptosis in response to stress [9]. Proteins were extracted from whole cells by using detergents and protease inhibitors [20]. For analysis of protein carbonylation, proteins were dissolved in 0.1 mol/L potassium phosphate buffer (pH 7.4) and carbonyls were derivatized using acetaldehyde-2,4-dinitrophenylhydrazine [21] before gel electrophoresis. Equal amounts of protein were resolved using 4–12% BisTris gels (Invitrogen, Carlsbad, CA) and electroblotted onto polyvinylidene difluoride membranes [20]. Proteins were probed using delipidated rabbit polyclonal anti-dinitrophenylhydrazine (Sigma, St. Louis, MO) and mouse monoclonal IgG₁ antihuman GADD153/CHOP (Santa Cruz Biotechnology, Santa Cruz, CA). Mouse antirabbit and goat antimouse IgG peroxidase conjugates were used as secondary antibodies (Sigma). Bands were visualized by chemiluminescence [20].

2.4. DNA damage

DNA breaks were quantified by comet assay (Trevigen, Gaithersburg, MD) as described [22]. This assay is based on the ability of cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid; strand breaks are associated with a “comet-like” appearance of dye (SYBR Green)-labeled DNA under the microscope. The comet tail (DNA damage) was quantified using the public domain National Institutes of Health Image program [23]; 50 cells from each treatment group were analyzed.

2.5. Cell cycle

For analysis of cell cycle distribution, DNA was labeled with propidium iodide and samples were analyzed using flow cytometry [24]. Flow cytometry experiments were conducted at the Cell Analysis Facility Center (University of Nebraska Medical Center, Omaha, NE).

2.6. DNA microarrays

Total RNA was extracted from HepG2 by using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). RNA from seven tissue culture flasks was pooled for each sample. RNA integrity and purity was confirmed using spectrometry and formaldehyde-agarose gel electrophoresis [25]. DNA microarray analysis was conducted as described [25], using the Human Genome U133A Array (Affymetrix, Santa Clara, CA). Data were

Table 1

Oligonucleotide primers used for reverse transcriptase polymerase chain reaction

Gene	Primer
glyc	5'-ATAGCCTCCAACACCCAGA-3' 5'-ATTCGGCCTTCATTTCTT-3'
cdc14	5'-AGCCCGTTTACCAACCTCT-3' 5'-TGGTGTGCTCCTCTGTCTTG-3'
bmp	5'-TTCCGGACTACATGCGGGATCTT-3' 5'-TGTCACATTGTGGTGACCAGTCT-3'
igf2	5'-TCCTCCCTGGACAATCAGAC-3' 5'-AGAAGCACCAGCATCGACTT-3'
plagl-1	5'-ACCGTCCAAAAACAGACT-3' 5'-AGAAACTTGGCAGGGATA-3'
gadh	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCCTGTTGCTGTA-3'

glyc, cartilage glycoprotein-39 (GenBank accession no. M80927.1); cdc14, cell division cycle 14 homolog A (GenBank accession no. NM_003672.1); bmp, bone morphogenetic protein 4 (GenBank accession no. D30751); igf2, insulin-like growth factor 2 (GenBank accession no. NM_000612.2); plagl-1, pleiomorphic adenoma gene-like 1 (GenBank accession no. NM_002656.1); gadph, glyceraldehyde-3-phosphate dehydrogenase (GenBank accession no. AF261085).

normalized using Microarray Suite 5.0, Micro DB, and Data Mining Tool 3.0 software (Affymetrix) as described [25]. Genes showing altered expression were categorized on the basis of their reported or suggested biological functions using Onto-Express [26]. Genes that were not annotated or not easily classified were excluded from the functional clustering analysis.

2.7. Reverse transcriptase–polymerase chain reaction

Expression profiles of selected riboflavin-dependent genes from microarray experiments were confirmed by

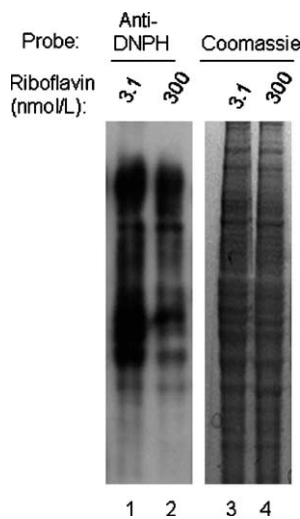


Fig. 1. Carbonylation of proteins increases in response to riboflavin deficiency in HepG2 cells. Cells were cultured in media containing 3.1 and 300 nmol/L riboflavin for 4 days. Carbonylated proteins were derivatized using acetaldehyde-2,4-dinitrophenylhydrazine and were visualized by Western blot analysis (lanes 1 and 2, anti-DNPH). Equal loading of lanes was confirmed by staining with Coomassie blue (lanes 3 and 4).

reverse transcriptase–polymerase chain reaction (RT-PCR). Analyses were run at least in duplicate [27] using the customized primers (Integrated DNA Technologies, Coralville, IA) listed in Table 1. The expression of glyceraldehyde-3-phosphate dehydrogenase was used as a control. Only values from within the exponential phase of PCR amplification were considered for analysis by gel densitometry [25].

2.8. Statistical analysis

Paired *t* test was used to determine whether differences were statistically significant between treatment groups [28]. 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 \pm S.D.

3. Results

3.1. Glutathione reductase

The activity of glutathione reductase in HepG2 cells depended on the concentration of riboflavin in culture media. The activities were “not detectable” and the units of absorbance was 0.09 ± 0.02 at 340 nm/(mg protein \times 12 min) in cells cultured in media containing 3.1 and 300 nmol/L riboflavin, respectively ($P < .05$; $n = 3$). This is consistent with cellular riboflavin depletion in response to riboflavin-deficient culture conditions. In previous studies we have shown that a reduced activity of glutathione reductase is associated with a low abundance of reduced glutathione [5].

3.2. Protein carbonylation

Riboflavin deficiency was associated with oxidative damage to cell proteins as judged by carbonylation of amino acid residues. Semiquantitative analysis of immunoblots (Fig. 1, lanes 1 and 2) by gel densitometry suggested that carbonylation of proteins was about 265% greater in riboflavin-deficient cells compared with riboflavin-supplemented controls. Equal loading of lanes was confirmed by staining of proteins using Coomassie blue (lanes 3 and 4).

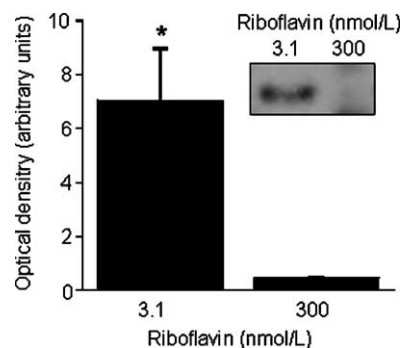


Fig. 2. Abundance of GADD153 increases in response to riboflavin deficiency in HepG2 cells. Cells were cultured in media containing 3.1 and 300 nmol/L riboflavin for 4 days. GADD153 was quantified using Western blot analysis and gel densitometry. $*P < .05$ vs. 300 nmol/L riboflavin ($n = 4$). The inset depicts a representative image from Western blot analysis.

3.3. DNA damage

Riboflavin deficiency was associated with increased incidence of DNA strand breaks as judged by comet assay. The tail moment was 5.2 ± 1.5 arbitrary units in riboflavin-deficient cells compared with 1.6 ± 0.5 arbitrary units in riboflavin-sufficient cells ($P < .05$; $n = 50$). As a positive control, cells were treated with 100 $\mu\text{mol/L}$ hydrogen peroxide for 1 h, a treatment known to cause DNA strand breaks [29]. The tail moment in these control cells was 5.7 ± 1.8 arbitrary units.

3.4. GADD153

The increased damage to proteins and DNA in riboflavin-deficient cells was associated with activation of GADD153. The abundance of GADD was about 14 times greater in riboflavin-deficient cells compared with riboflavin-sufficient controls (Fig. 2). Equal loading of lanes on electrophoresis gels was confirmed by staining of proteins with Coomassie blue (data not shown).

3.5. Cell cycle

Riboflavin-deficient cells arrested in G1 phase of the cell cycle. Specifically, analysis by flow cytometry suggested that

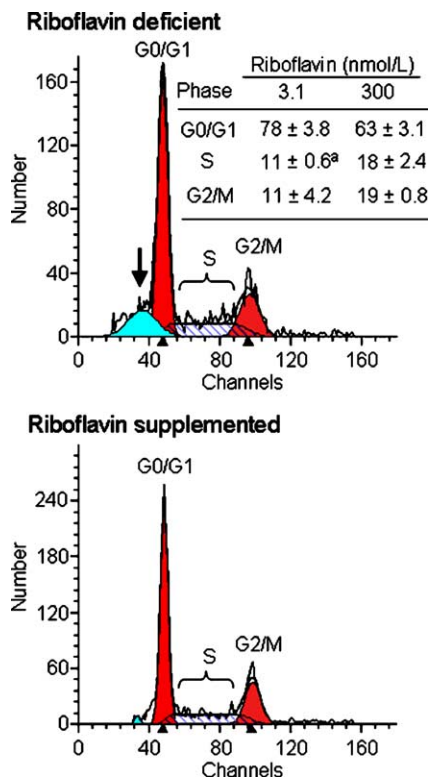


Fig. 3. Riboflavin deficiency causes G1 phase arrest in HepG2 cells. Cells were cultured in media containing 3.1 and 300 nmol/L riboflavin for 4 days. Cell cycle distribution was quantified by flow cytometry. Representative flow cytometry charts are depicted; the arrow denotes apoptotic cells in the riboflavin-deficient group. The insert provides a table with percentage of cell cycle distributions. ^a $P < .05$, significantly different from riboflavin-sufficient controls ($n = 3$).

Table 2

Riboflavin-responsive genes in HepG2 cells

Treatment group	Gene expression compared with physiological control	
	Increase	Decrease
	Gene (relative abundance of mRNA) ^a	
Deficient	ILGF7 (3475%)	PCAM (20%)
	CDC14 (3230%)	PSTK (20%)
	CPC8 (1638%)	HCAG (25%)
	HEMBA1000568 (1292%)	SCF172 (25%)
	HPF/1J11798 (1199%)	KIAA0786 (37%)
Supplemented	HDCR (1500%)	ADBCTC02 (14%)
	Xr78h03 (1392%)	DLHSP (31%)
	ILGF2 (1031%)	MSL (38%)
	TXN d3 (884%)	RPL23 (41%)
	IGF-II (884%)	SPF45 (61%)

ADBCTC02, clone (GenBank accession no. AU143984); CDC14, cell division cycle 14 (GenBank accession no. NM_003672.1); CPC8, complement protein C8 gamma subunit (GenBank accession no. M17263.1); DLHSP, DnaJ heat shock protein 40 (GenBank accession no. NM_007034.2); HCAG, hepatocellular carcinoma-associated gene (GenBank accession no. NM_018687.1); HDCR, Huntington's disease candidate region (GenBank accession no. L37198.1); HEMBA1000568, *Homo sapiens* cDNA clone (GenBank accession no. AU143984); HPF/1J11798, hypothetical protein LFJ11798 (GenBank accession no. NM_024907.1); ILGF7, insulin growth factor binding protein 7 (GenBank accession no. NM_001443.1); ILGF2, insulin-like growth factor 2 (somatomedin A) (GenBank accession no. NM_000612.2); KIAA0786, latrophilin (GenBank accession no. NM_012302.1); MSL, mannose-specific lectin (GenBank accession no. U09716.1); PCAM, platelet endothelial cell adhesion molecule (GenBank accession no. aaA702701); IGF-II, pre-proinsulin-like growth factor II (GenBank accession no. M1786); PSTK, protein-serine threonine kinase (GenBank accession no. Z25422.1); RPL23, ribosomal protein 23 (GenBank accession no. NM_000978.1); SCF172, solute carrier family 17 (sodium phosphate) member 2 (GenBank accession no. NM_005835.1); SPF45, splicing factor (45 kDa) (GenBank accession no. NM_006450.1); xr78h03, *H. sapiens* cDNA clone (GenBank accession no. BE138674); TXN d3, thioredoxin delta 3 (GenBank accession no. AF065241.1).

^a Cells cultured in physiological medium (12.6 nmol/L of riboflavin) were used as a reference. mRNA abundance is shown in parentheses, using cells in physiological medium as control (=100%).

78% of riboflavin-deficient cells were in G1 phase of the cell cycle, but only 63% of riboflavin-supplemented controls were in G1 phase (Fig. 3). The relative abundance of cells in S phase of the cycle decreased from 18% to 11% in response to riboflavin deficiency. Note the presence of apoptotic cells in the riboflavin-deficient treatment group (Fig. 3).

3.6. Gene expression analysis

The concentration of riboflavin in culture media affected the abundance of mRNA encoding 2232 genes, as judged by DNA microarray analysis. Genes were considered riboflavin-responsive if the abundance of mRNA in a given treatment group (riboflavin-deficient or riboflavin-supplemented) changed by at least 40% compared with physiological controls cultured in medium containing 12.6 nmol/L riboflavin. If cells were cultured in riboflavin-deficient medium, the expression of 742 and 578 genes increased and decreased, respectively, compared with physiological controls. If cells were cultured in

riboflavin-supplemented medium, the expression of 567 and 345 genes increased and decreased, respectively, compared with physiological controls.

Table 2 provides a list of those genes that were affected most dramatically by the concentrations of riboflavin in culture media, as judged by DNA microarray. The relative abundance of mRNA encoding these highly riboflavin-responsive genes is expressed in units of percentage of controls (cells cultured in physiological medium=100%). The relative abundance of mRNA encoding highly riboflavin-induced genes was 884–1500% in riboflavin-supplemented cells compared with physiological controls; the relative abundance of mRNA highly riboflavin-repressed genes was 14–61% in riboflavin-supplemented cells compared with physiological controls. The relative abundance of mRNA encoding highly riboflavin-induced genes was 3200–1576% in riboflavin-deficient cells compared with physiological controls; the relative abundance of mRNA highly riboflavin-repressed genes was 20–27% in riboflavin-deficient cells compared with physiological controls.

The relative abundances of mRNA as determined by DNA microarray were independently confirmed by RT-PCR as follows. First, we selected the following four riboflavin-responsive genes from our DNA microarray experiments: insulin-like growth factor 2 (increased expression in response to riboflavin supplementation), cell division cycle 14 homolog A (increased expression in response to riboflavin deficiency), cartilage glycoprotein-39 (increased expression in response to riboflavin deficiency), and bone morphogenetic protein 4 (decreased expression in response to riboflavin supplementation). Next, the abundance of mRNA encoding these four genes was quantified by RT-PCR. Comparative analysis of mRNA abundance by DNA microarrays and RT-PCR produced the same trends for all four genes (Fig. 4). For example, both DNA

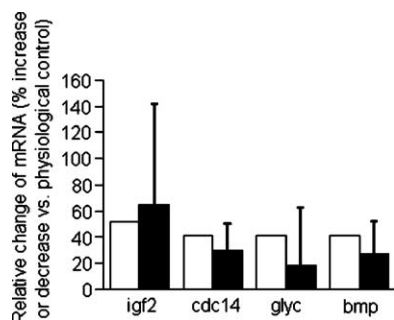


Fig. 4. Comparative analysis of gene expression by DNA microarrays and RT-PCR in HepG2 cells. Abundances of mRNA encoding the following four genes were quantified: bmp, bone morphogenetic protein 4 (GenBank accession no. D30751); cdc14, cell division cycle 14 homolog A (GenBank accession no. NM_003672.1); glyc, cartilage glycoprotein-39 (GenBank accession no. M80927.1); igf2, insulin-like growth factor 2 (GenBank accession no. NM_000612.2). Percentage of changes may refer to decreased or increased abundance of mRNA in cells cultured in media containing 3.1 or 300 nmol/L riboflavin compared with physiological controls (see text). Open bars, DNA microarray; black bars, RT-PCR ($n=4-5$ for RT-PCR).

Table 3
Clusters of riboflavin-dependent genes in HepG2 cells

Gene cluster	Transcriptional response to culture conditions			
	Increased transcription ^a		Decreased transcription	
	Def.	Suppl.	Def.	Suppl.
	Number of genes (<i>n</i>)			
Protein synthesis or degradation	22	42	54	31
Cell cycle progression and proliferation	89	43	35	32
Apoptosis	40	13	6	9

Def., riboflavin-deficient medium (3.1 nmol/L of riboflavin); suppl., riboflavin-supplemented medium (300 nmol/L of riboflavin).

^a Compared to cells cultured in physiological control medium (12.6 nmol/L of riboflavin).

microarrays and RT-PCR identified insulin-like growth factor 2 as a gene that was up-regulated in response to pharmacological concentrations of riboflavin. Moreover, the magnitude of effects was similar if quantified by DNA microarray and RT-PCR (Fig. 4). The abundance of mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (control) was not affected by riboflavin (data not shown). Collectively, these data suggest that the data collected by using DNA microarrays are both accurate and precise.

Riboflavin-dependent genes were not randomly distributed in the HepG2 cell genome but were assigned to gene clusters. Three predominant clusters of riboflavin-dependent genes were identified if classification was based on the biological function of gene products. These clusters comprised gene products participating in protein synthesis or degradation, cell cycle progression and proliferation, and apoptosis; numerous minor clusters accounted for the balance (Table 3).

The cluster of apoptosis-related genes is important in the light of protein and DNA damage in response to riboflavin deficiency. The cluster of apoptosis-related genes activated by riboflavin-deficiency included *pleiomorphic adenoma gene-like 1* and *perforin-1*. The abundance of mRNA coding for select apoptosis-related genes was confirmed by RT-PCR. For example, the abundance of mRNA encoding the pleiomorphic adenoma gene-like 1 protein increased by 240% and 183% in riboflavin-deficient cells as judged by RT-PCR and DNA microarray, respectively.

4. Discussion

The present study provides evidence that riboflavin deficiency in HepG2 cells (i) decreases activities of glutathione reductase, (ii) is associated with oxidative damage (carbonylation) to proteins, (iii) increases the incidence of DNA strand breaks, (iv) causes G1 phase arrest and (v) is associated with increased expression of clusters of genes that play roles in cell stress and apoptosis.

These findings have practical implications for nutritionists based on the following. First, the concentration of riboflavin in deficient medium (3.1 nmol/L) is similar

to concentrations observed in plasma from moderately deficient pregnant women [15]. We shall point out that the applicability of the findings reported here to *in vivo* situations is somewhat uncertain. Note that liver cells are supplied with water-soluble nutrients through the portal vein *in vivo*, and that riboflavin concentrations are greater in portal blood compared with peripheral blood [17]. Hence, riboflavin concentrations in moderately deficient culture medium might be lower than concentrations pertinent to liver cells in moderately riboflavin-deficient individuals *in vivo*. Notwithstanding this uncertainty, the current study provides proof of principle, that is, moderate riboflavin impairs integrity of proteins and DNA, and cell proliferation. Second, effects of riboflavin deficiency on protein carbonylation, DNA breaks and cell proliferation were detectable after only 4 days in riboflavin-deficient medium. It is reasonable to assume that states of riboflavin deficiency typically exceed 4 days in humans. For example, riboflavin plasma concentrations may decrease in pregnancy [15] and in response to treatment with antimalarial drugs [1]. Moreover, evidence has been provided that hypothyroidism impairs the conversion of riboflavin to its coenzyme forms [30,31].

Effects of riboflavin deficiency on protein carbonylation, DNA strand breaks and cell cycle progression are biologically important. First, carbonylation of proteins in response to oxidative stress causes changes in protein conformation-mediated events such as myofibrillar proteolysis [32]. Second, DNA strand breaks cause chromosomal abnormalities. Cells containing damaged DNA are to be eliminated by apoptosis or DNA repair. Incomplete elimination of DNA damage by any of these mechanisms may lead to malignant transformation and clonal expansion of cells, that is, cancer. Third, cell cycle arrest in response to riboflavin deficiency may impair the function of tissues that depend on rapid cell proliferation such as the intestinal mucosa. However, it would be premature to conclude that riboflavin deficiency can be used to selectively inhibit tumor growth in humans.

Riboflavin deficiency is likely to affect biological processes other than glutathione-dependent prevention of oxidative damage. For example, riboflavin deficiency impairs the oxidative folding of proteins [4,5] by Ero1 and sulfhydryl oxidases [2,3], impairs genomic stability in human lymphocytes [33], causes anemia in humans [34] and also impairs other biological processes [35]. Many of these diverse effects of riboflavin deficiency may be associated with similar symptoms such as small cell proliferation rates.

The mRNA pattern in riboflavin-deficient cells is consistent with cell stress. For example, riboflavin deficiency was associated with an increased abundance of mRNA from gene clusters associated with protein synthesis or degradation, cell cycle progression and proliferation, and apoptosis. These results need to be interpreted with caution based on the following. Riboflavin deficiency is associated with a global decrease of translational activity and with a global increase of proteolytic activity, but also with a specific increase in the synthesis of some proteins [4,5]. Hence,

posttranscriptional events might be important confounders when analyzing effects of riboflavin on gene expression by using DNA microarrays. This uncertainty notwithstanding, gene expression data presented here clearly support our hypothesis that riboflavin deficiency causes cell stress.

Previous studies provided evidence that the sensitivity of cells for developing riboflavin deficiency is tissue-specific. For example, HepG2 liver cells develop signs of riboflavin deficiency even when riboflavin concentrations in culture media are only moderately low [5]. In contrast, Jurkat lymphoid cells are relatively resistant to developing signs of riboflavin deficiency even if cultured in severely deficient medium [4]. The reasons for these differences are uncertain. Likewise, it is unknown whether riboflavin deficiency in human subjects is associated with increased protein and DNA damage in tissues, mediating cell cycle arrest. Note that cell culture systems are artificial systems that might be associated with high oxygen tension, high levels of pro-oxidant metal ions and deficiency in antioxidants other than riboflavin. Collectively, future studies of riboflavin requirements and deficiency should include human or animal models and should take into account that some tissues have a greater demand for riboflavin than others.

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