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Research Articles

Photoreceptor damage following exposure to excess riboflavin

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Abstract. Flavins generate oxidants during metabolism and when exposed to light[1]. Here we report that the photoreceptor layer of retinas from black-eyed rats is reduced in size by a dietary regime containing excess riboflavin. The effect of excess riboflavin was dose-dependent and was manifested by a decrease in photoreceptor length. This decrease was due in part to a reduction in the thickness of the outer nuclear layer, a structure formed from stacked photoreceptor nuclei. These changes were accompanied by an increase in photoreceptor outer segment autofluorescence following illumination at 328 nm, a wavelength that corresponds to the excitation maxima of oxidized lipopigments of the retinal pigment epithelium[2].
Key words. Riboflavin; flavins; tissue damage; retina; retinal degeneration.

The major flavins in the retina are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin[3]. They occur in the relative proportion of 65%, 32% and 3% respectively[4,5]. FAD and FMN participate in the transfer of electrons between their isoalloxazine nucleus and other reactants in cellular reactions where they function as coenzymes. A variety of cellular oxidants are generated by flavoenzymes during metabolism[6]. Flavoenzymes include oxidases, hydrogenases and monoxygenases and are primarily located in mitochondria, peroxisomes and microsomes[7,8]. During the oxidation of substrate, flavin oxidases (e.g. glucose oxidase) react rapidly with O_2 and produce a high yield of H_2O_2. Dehydrogenases (e.g. fatty acyl-CoA dehydrogenase) and reductases (e.g. glutathione reductase) accept two electrons from substrates and then undergo reoxidation one electron at a time through the semiquinone state. During reoxidation, if O_2 is used as an electron acceptor, superoxide is formed[7]. In addition to the generation of metabolic oxidants, flavins are readily photobleached over a broad spectrum that includes UV, UV-B, UV-A and visible light. Photoactivated flavins undergo photoreduction, photodegradation, and in the presence of O_2 generate H_2O_2, singlet oxygen ($^1O_2$) and superoxide radicals[9]. DNA serves as a good reducing substrate for photoactivated riboflavin. The planar riboflavin molecule intercalates between the stacked bases at the center of the DNA double helix and the guanine base of GMP reacts with the flavin isoalloxazine ring[10]. Photoactivation leads to single strand breaks due to opening of the guanine ring and the generation of radicals[11]. Photoactivated riboflavin has been reported to degrade bacteriophage[12], tobacco mosaic virus[13], *E. coli*[14], and cultured human cells[15]. These observations lead us to question whether riboflavin toxicity occurred in the most light-focused and metabolic tissue in the body, the retina.

Materials and methods

Black-eyed Long Evans rats were obtained from Charles Rivers Laboratories, Worthington, MA. Rats were housed in hanging stainless steel cages with solid metal sides and wire fronts and bottoms. Racked cages were placed in a room with controlled temperature, humidity and cyclic light (6 a.m.–6 p.m. light/6 p.m.–6 a.m. dark). The illumination at the front of the cages was 3–10 lux. The diets contained all other nutrients at recommended levels except for vitamin E, which was present at twice the recommended concentration[16,17], and riboflavin which was varied as described. In order to minimize the exposure of dietary riboflavin to light, rats were provided access to food only during the dark cycle. Rats were killed by an overdose of carbon dioxide. For morphological evaluation, eyes were removed and the posterior section of the globe embedded in epoxy resin for 1-micrometer thick sections using methods previously described[18].

For spectrofluorometric evaluation the posterior segments of eyes from black-eyed rats, provided 6 and 12 mg riboflavin/kg diet for 30 d, were embedded in polyester wax for 10-micrometer sections using methods previously described[18]. Unstained sections were evaluated. The intensity of fluorescence over the photoreceptor outer segments was measured using a Farrand microspectrofluorometer equipped with Zeiss excitation filters with maximum at 328 nm or at 373 nm. Emission fluorescence of outer segments was corrected for background fluorescence by subtraction of values obtained

through adjacent sections of the glass mount without tissue. A dichroic mirror and barrier filter (510 nm) used for epifluorescent illumination on the fluorescence microscope was used for the measurement of light emission above 510 nm.

Experimental protocols were carried out as follows. In the 30-day protocol, 16 rats were divided into 2 groups of 8. One group was provided a control diet containing the recommended level of dietary riboflavin (6 mg riboflavin/kg diet)[16,17]. The other group was provided the excessive level of 12 mg/kg. Animals were provided the diets for 30 d. In the 90-day protocol, 24 rats were divided into 3 groups of 8. The groups were provided 3, 12 or 30 mg riboflavin/kg diet for 90 d.

Results and discussion

The retinas of rats provided with excessive riboflavin (12 mg/kg) in the 30-day protocol showed a reduction in rod photoreceptor length (outer nuclear layer + inner and outer segments). The inclusive outer nuclear layer, containing the stacked photoreceptor nuclei, was reduced in rats provided with excessive riboflavin (fig. 1a and b). To assess whether these changes were dose-dependent we provided 3 groups of 8 rats with diets containing 6, 12 or 30 mg riboflavin/kg diet. Retinas were examined after 90 d. Morphological evaluation demonstrated an inverse relationship between dietary riboflavin concentration and both photoreceptor length and outer nuclear layer thickness (fig. 2). Examination of the outer nuclear layer showed that these changes

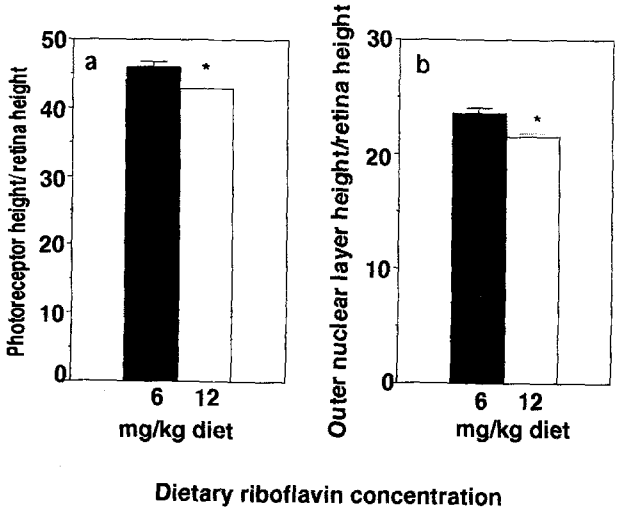

Figure 1. Long Evans black-eyed rats were provided with the recommended level of riboflavin (6 mg/kg diet) or an excessive amount (12 mg/kg) for 30 days. Each bar represents results from 8 rats ± SEM (*$p < 0.01$). *a* Total photoreceptor layer (outer nuclear layer + inner segment layer + outer segment layer) height and *b* outer nuclear layer thickness are expressed as a ratio of the height of the retina to control for shrinkage artifact. Excessive riboflavin consumption resulted in a decrease in both photoreceptor length and outer nuclear layer thickness.

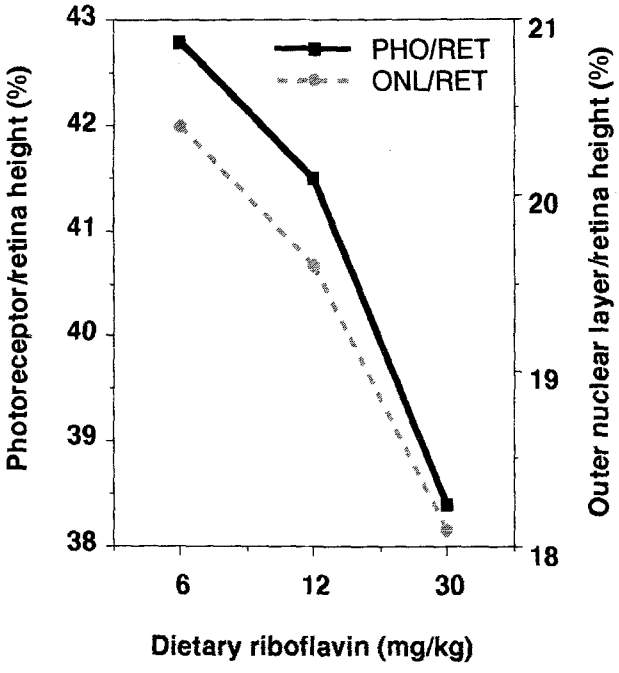

Figure 2. A dose-response experiment was undertaken to assess the relationship of damage to the level of riboflavin intake. Long evans black-eyed rats were provided 6, 12 or 30 mg riboflavin/kg diet for 3 months. Each point represents results from 8 rats. The height of both the total photoreceptor layer (outer nuclear layer + inner segment layer + outer segment layer) and the outer nuclear layer are expressed as a ratio of the height of the retina to control for shrinkage artifact. The dose-dependency for 6 to 30 mg riboflavin/kg was significant ($p < 0.01$) for both the photoreceptor and outer nuclear layers.

were due in part to the loss of photoreceptors (fig. 3). Oxyradicals generated by flavins in the presence of fatty acids can also lead to the formation of flavin-fatty acid esters[20]. Lipid peroxidation increases with age in the retina[21] and is associated with an accumulation of lipofuscin fluorophores[22]. We assessed the accumulation of fluorophores in our animals by evaluating autofluorescence of the rod photoreceptor outer segments. For this we used microspectrofluorometry to evaluate unstained paraffin embedded sections of retinas obtained from rats provided with 6 and 12 mg riboflavin/kg diet for 30 d. Autofluorescence was significantly greater over the photoreceptor outer segments of rats provided with excess riboflavin (fig. 4). The fluorescence occurred following excitation at 328 nm, a wavelength near the excitation maxima of several green (328 nm, 330 nm), yellow-green (330 nm, 331 nm) and golden-yellow (330 nm) emitting fluorophores that have been identified in the retinal pigment epithelium[2]. Fluorescence did not develop following excitation at 364 nm, a wavelength near the 373 nm absorption peak of riboflavin (fig. 4).

Oxidative injury has been implicated in the pathogenesis of numerous degenerative diseases[24]. Among these is the gradual loss of photoreceptors in the macula in age-related macular degeneration, a disease that represents

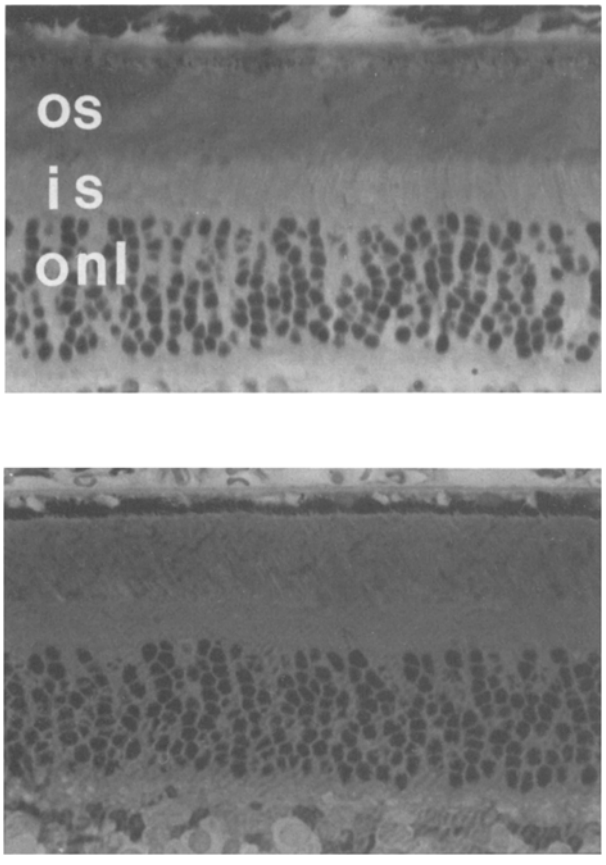

Figure 3. Light micrographs of sections of superior retinae from black-eyed Charles Rivers Long Evans rats showing the photoreceptor outer segments (OS), inner segments (IS) and outer nuclear layer (ONL). Top: region from rat provided with an excessive concentration riboflavin (30 mg/kg) for 90 days. The outer nuclear layer is thinner than controls with empty areas of missing rod photoreceptor nuclei. Bottom: region from a control rat provided with the recommended riboflavin level (6 mg/kg) for 90 days. Toluidine blue stain. Magnification 444×.

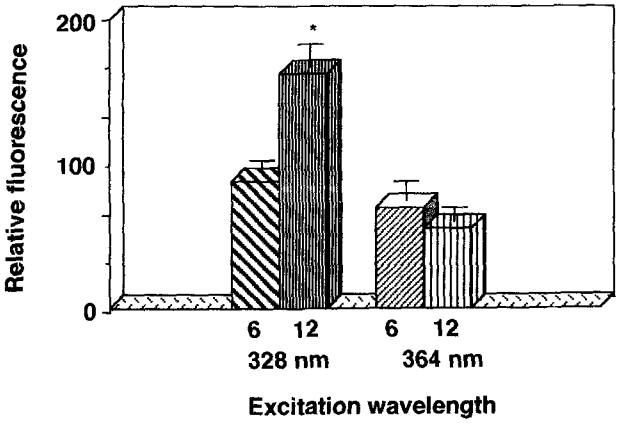

Figure 4. The relative fluorescence over the rod photoreceptor outer segment region of rats provided with the recommended level of dietary riboflavin (6 mg/kg) or excess (12 mg/kg) for 30 days. The autofluorescence was greater from rats provided with the excessive level of riboflavin when the outer segments were excited near the excitation of retinal fluorophores (328 nm) but not 364 nm, a wavelength near the 373 nm absorption peak of riboflavin.

the leading cause of irreversible visual loss in the United States and United Kingdom in persons over 50 years old[24]. A recent retrospective epidemiological study has shown that multivitamin supplementation, caloric intake and plasma vitamin A levels are all positively associated with age-related macular degeneration while the plasma antioxidants, vitamins C, E and beta-carotene are protective[25]. These observations may simply indicate that once individuals has been informed they have macular degeneration they then begin taking multivitamin supplements as a preventive health measure. Our observations suggest it would be prudent to evaluate riboflavin supplementation as a possible risk factor in the pathogenesis of this and other environmentally related degenerative diseases of the retina in prospective epidemiological studies. Evaluation of a cross-section of persons using multivatimin supplements has shown they have an intake of riboflavin that is 36 times the U.S. recommended daily allowance[26]. The recommended allowance is 0.6 mg/1000 kcal of food consumed or 1.3 and 1.7 mg/day respectively for adult women and men[27]. In women and men over 51 yrs the amount recommended is decreased to 1.2 and 1.4 mg/day due to the lowered energy intake of older populations[27].

Flavins have long been implicated in a variety of photobiological processes including photodinesis and phototropism in plants[28] and photomorphogenesis in insects[29]. Could our results point to a physiological role for retinal flavins based on their photobiological properties? It is possible that the excessive riboflavin in our studies resulted in an aberrant manifestation of the use of flavins enjoined as a photosensitive trigger. The light exposure in our studies was well above the extremely low light levels (0.002 lux) required to induce disk shedding[30]. The broad absorption spectrum of flavins (maxima for excitation: 224, 260, 373, and 445 nm) and their ability to initiate comformational changes in associated proteins make them candidates for transmitting photic zeitgeber signals to phase shift ocular circadian rhythms[30]. The list of retinal events with light-elicited properties continues to grow and includes stimulation of inner segment synthesis, transcription of inner segment mRNA, activation of lysosomal enzymes in the pigment epithelium and shifts in molecular conformation and distribution of the interphotoreceptor matrix[30]. The visual pigments undoubtedly serve as the photon acceptors for many of these events. The results of the present study demonstrate that in addition to vitamin A, at least one other photosensitive compound obtained from the food supply, riboflavin, impacts the visual system. This leads to the question of whether other photosensitive compounds from the environment may do the same.

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