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ENHANCED DEPLETION OF LENS REDUCED GLUTATHIONE BY ADRIAMYCIN® IN RIBOFLAVIN-DEFICIENT RATS

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Abstract—The anticancer drug Adriamycin® has photosensitizing properties which potentially may be detrimental to lens tissue. Since reduced glutathione (GSH) serves to protect lens from photo-oxidative stress and dietary riboflavin is required by glutathione reductase to regenerate GSH, we investigated whether Adriamycin® intensifies the depletion of GSH levels in rat lens during dietary riboflavin deficiency. Three-week-old rats were divided into two groups. One group was fed a diet deficient in riboflavin (<1 ppm) and the other group was pair-fed a control diet containing adequate riboflavin (8.5 ppm). After 6–12 weeks of dietary treatment, half the animals in each dietary group received Adriamycin® (8 mg/kg/day) intraperitoneally for 3 days. After killing the rats, lenses were removed, and GSH content and glutathione reductase activity were measured in freshly prepared homogenates. To determine the extent of systemic oxidative stress and the degree of riboflavin deficiency, glucose-6-phosphate dehydrogenase and glutathione reductase activities, respectively, were measured in erythrocytes. In lens of rats fed the riboflavin-sufficient diet, treatment with Adriamycin® did not diminish GSH content or alter glutathione reductase activity. In confirmation of reports by others, lenses of animals fed the riboflavin-deficient diet had diminished GSH levels, lower basal glutathione reductase activity, and elevated glutathione reductase activity coefficients compared to those of animals pair-fed the control diet. The present study shows that in riboflavin-deficient rats, Adriamycin® exacerbated the depletion of GSH but did not reduce further glutathione reductase activity. The implications of these findings are that nutritional deficiencies, in particular riboflavin deprivation, may pose a potential risk to lenticular tissue following Adriamycin® treatment.

Over 95% of the protein composition of lens tissue is composed of crystallins [1]. Since crystallins turn over very slowly, denatured products of this specialized protein induced by ultraviolet light and/or photosensitizing drugs tend to accumulate. Photo-oxidative damage to lens results in eventual formation of cataracts [2], the predominant cause of impaired vision and blindness in the elderly.

Under normal circumstances, the lens possesses efficient non-enzymatic and enzymatic antioxidative systems. Vitamin C and vitamin E are present in adequate concentrations in the lens, and their presence suggests a protective function against photo-oxidative damage [2, 3]. Lenses also contain superoxide dismutase and catalase, the activities of which decrease with age and cataractogenesis [4, 5]. Another major antioxidative constituent of lens is the tripeptide, reduced glutathione (GSH) [6]. Glutathione peroxidase requires GSH to prevent peroxidative damage to crystallin proteins as well as cell membranes of lens. Glutathione reductase, a flavin adenine dinucleotide (FAD) containing enzyme, is involved in regenerating GSH from oxidized glutathione (GSSG). Both the concentration of GSH and the activities of glutathione reductase and glutathione peroxidase are reported to be diminished in cataractous lens [6, 7]. Thus, a critical deficit in riboflavin may potentially leave lens tissue vulnerable to oxidative injury.

Riboflavin deficiency has been associated with cataract formation in some studies [8–10], whereas others have failed to confirm this observation [11–13]. Yagi and coworkers [14] recently found that riboflavin deficiency, even in the presence of adequate levels of dietary vitamin E, increases serum lipid peroxide concentration which correlates positively with cataract formation in rats. However, Srivastava and Beutler [7] showed that after 21 days of feeding rats a riboflavin-deficient diet, glutathione reductase activity of lens was reduced to 75% of normal chow-fed animals. This deficit in activity of glutathione reductase did not alter the concentration of GSH or the lenticular transparency. Presently, it is unknown whether the depletion of GSH or the damage to lens by a photo-oxidative agent becomes progressively worse during riboflavin deficiency.

Adriamycin® is an anthracycline antibiotic which is used extensively for the treatment of a wide variety of human malignancies. In spite of its effectiveness as a chemotherapeutic agent, Adriamycin® has a number of serious side effects, notably cardiac and skeletal myopathies [15] and nephrotic syndrome [16]. Anthracycline derivatives also have photosensitizing properties which could be detrimental particularly to lens tissue during therapy [17]. Furthermore, our laboratory has reported previously that Adriamycin® produces a dose-related decrease

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in FAD formation in various tissues in rats [18]. As a result of this decline in tissue FAD levels, chronic low-dose administration of this drug produces a small but significant rise in the glutathione reductase activity coefficient (GRAC) in erythrocytes, indicating a marginal riboflavin deficiency [19]. Since dietary riboflavin deficiency diminishes the activity of erythrocyte glutathione reductase and results in elevation of the GRAC [20], we determined whether administration of Adriamycin® to riboflavin-deficient animals compromises further their riboflavin nutriture and exacerbates depletion of GSH in lens tissue.

METHODS

Chemicals, reagents, and drugs. Adriamycin® was purchased from Adria Laboratories (Columbus, OH) as a crystalline powder and reconstituted with saline prior to injection into animals. Bicinchoninic acid (BCA) reagent for protein assay was obtained from the Pierce Chemical Co. (Rockford, IL). Reagents for glucose-6-phosphate dehydrogenase assay, FAD, $NADP^+$, NADPH, GSH, GSSG, and 5,5′-dithiobis-2-nitrobenzoic acid were purchased from the Sigma Chemical Co. (St. Louis, MO).

Diets and treatment of animals. Diets used in this study were prepared by Dyets, Inc., Bethlehem, PA. Three-week-old male Holtzman rats (Holtzman Co., Madison, WI) were divided into two experimental groups, designated control and riboflavin-deficient, and were housed individually in metabolic cages. Animals in the riboflavin-deficient group were fed a diet which was analyzed in our laboratory and found to contain less than 1 μg of riboflavin/g diet. Controls were pair-fed a diet identical in composition but supplemented with 8.5 μg riboflavin/g diet, which represents three times the Recommended Dietary Allowance (RDA) for riboflavin in the rat and approximately four to five times that needed to achieve maximal growth of young male rats. After 6–12 weeks on diets, half the number of rats from each dietary group received (intraperitoneally) Adriamycin® (8 mg/kg/day, 24 mg/kg cumulative dose) for 3 days. The other half received an equivalent volume of saline. On the day following the last injection (day 4), rats were anesthetized by exposing them to solid carbon dioxide. Blood samples were drawn by cardiac puncture into tubes containing EDTA as an anticoagulant. Following extraction of the eyes, lenses were removed carefully via an excision in the posterior segment.

Reduced glutathione (GSH) concentration. Reduced glutathione was measured by the method of Beutler [21]. Briefly, the lens was homogenized at 0° in 10 vol. of water, and an aliquot of the homogenate was added to a precipitation solution, containing metaphosphoric acid, EDTA, and sodium chloride. After standing for 5 min, the mixture was centrifuged at 5000 *g* for 10 min, and an aliquot of the supernatant fraction was added to 0.3 M Na_2HPO_4. An initial absorbance reading was made at 412 nm followed by a second at the same wavelength after the addition of the sulfhydryl reagent, 5,5′-dithiobis-2-nitrobenzoic acid. The final concentration of GSH was reported as nanomoles per milligram protein.

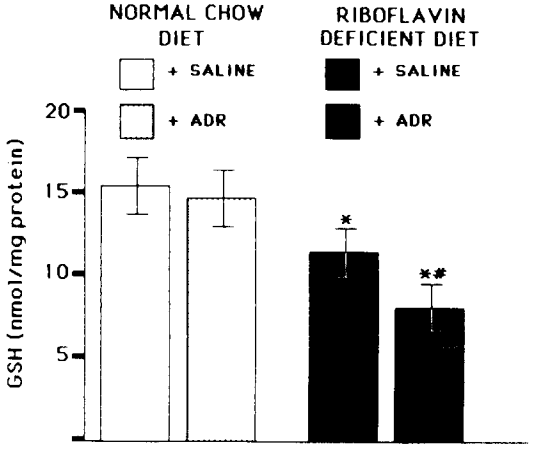

Fig. 1. Effects of riboflavin deficiency and Adriamycin® treatment on concentration of reduced glutathione in lens. For details of preparation of riboflavin-deficient animals and drug treatment, see Methods. Values are means ± SE of at least 13 independent observations. Key: (*) $P < 0.05$ versus control animals; and (#) $P < 0.05$ versus saline-treated riboflavin-deficient animals.

Glutathione reductase activity and activity coefficient (GRAC). The basal activity of glutathione reductase and the GRAC which is the stimulated activity of the enzyme in the presence of exogenous FAD were determined in lens homogenates by using the method of Beutler [22]. In erythrocytes, GRAC was determined as a measure of riboflavin nutritional status. In this report, we define as riboflavin deficient those animals that have an erythrocyte GRAC equal to or greater than 1.7.

Glucose-6-phosphate dehydrogenase assay. Glucose-6-phosphate dehydrogenase activity was assayed spectrophotometrically at 340 nm by measuring the rate of formation of NADPH in the presence of glucose-6-phosphate [23].

Protein assay. Protein concentration was determined by incubating diluted protein samples with BCA reagent at 37° for 30 min [24]. The spectrophotometric quantitation of protein was conducted by determining the absorbance at 562 nm.

Statistical analysis. For statistical analysis, we used Student's *t*-test for paired analysis to compare results in samples from riboflavin-deficient and control animals. A P value of less than 0.05 was considered significant. All results are shown as the mean ± SE.

RESULTS

GSH concentration. As illustrated in Fig. 1, rats fed a riboflavin-sufficient diet and treated with Adriamycin® did not show diminished lens glutathione content. Conversely, animals fed the riboflavin-deficient diet had diminished GSH levels (74% of control value). In riboflavin-deficient rats, Adriamycin® exacerbated the depletion of glutathione. The concentration of GSH in Adriamycin®-treated, riboflavin-deficient animals was 52% of control values.

Table 1. Lenticular glutathione reductase activity and activity coefficient in riboflavin-sufficient and riboflavin-deficient rats

Treatment group	Glutathione reductase	
	Activity (units/min)	Activity coefficient
Control	0.32 ± 0.03	1.28 ± 0.19
Control + Adriamycin®	0.28 ± 0.03	1.30 ± 0.39
Riboflavin-deficient	0.19 ± 0.02*	1.77 ± 0.32*
Riboflavin-deficient + Adriamycin®	0.18 ± 0.03*	1.60 ± 0.31*

Values are means ± SE; the number of animals in each study group varied from nine to twelve.
* $P < 0.01$ vs control.

Table 2. Glucose-6-phosphate dehydrogenase (G-6-PDH) activity and glutathione reductase activity coefficient (GRAC) in rat erythrocytes

Treatment group	G-6-PDH activity (units/mg Hb)	GRAC
Control	18.90 ± 0.62	1.51 ± 0.32
Control + Adriamycin®	18.22 ± 0.80	1.44 ± 0.17
Riboflavin-deficient	18.72 ± 0.47	2.60 ± 0.59*
Riboflavin-deficient + Adriamycin®	18.96 ± 0.92	2.08 ± 0.87†

Values are means ± SE; there were twelve animals per treatment group.
* $P < 0.002$ vs control.
† $P < 0.05$ vs control + Adriamycin®.

The mean ± SE for GSH in control animals, shown in Fig. 1, was 15.45 ± 1.66 nmol/mg protein.

Glutathione reductase and glutathione reductase activity coefficient. Table 1 shows that Adriamycin® administration did not alter either the basal activity or the activity coefficient of glutathione reductase in the lens of riboflavin-sufficient rats. Riboflavin deficiency reduced the basal activity of glutathione reductase and resulted in an elevated activity coefficient of glutathione reductase. Adriamycin® treatment did not reduce further glutathione reductase activity in the riboflavin-deficient animals. The glutathione reductase activity coefficient also did not increase due to Adriamycin® administration in these animals.

Table 2 indicates that the erythrocyte GRAC, which is a measure of riboflavin nutritional status, was elevated significantly in riboflavin-deficient animals regardless of Adriamycin® treatment. In addition, Adriamycin® did not change further the erythrocyte GRAC in animals fed either the riboflavin-deficient or riboflavin-sufficient diet. The erythrocyte GRAC of pair-fed control rats in this experiment was slightly higher than that normally observed in riboflavin-sufficient animals (range 1.0 to 1.3).

Glucose-6-phosphate dehydrogenase activity. As illustrated in Table 2, during the time course of this experiment, neither riboflavin deficiency nor Adriamycin® treatment, either alone or in combination, altered the activity of erythrocyte glucose-6-phosphate dehydrogenase.

DISCUSSION

A number of studies have reported that animals fed a riboflavin-deficient diet frequently develop cataracts [8–10]. However, studies by other investigators have not confirmed these findings [11–13]. The differences in results were attributed to the duration and degree of riboflavin deficiency as well as to the effects of various endogenous and exogenous contributing factors, e.g. other nutritional deficiencies, aging, ultraviolet light, and photoreactive drugs. The mechanism responsible for cataract formation or impaired vision in riboflavin deficiency is not known with certainty. Riboflavin maintains lens GSH levels by optimizing the activity of glutathione reductase, an FAD-requiring enzyme [20]. Riboflavin deficiency has been associated with the reduction in lens of a number of vital intracellular components, e.g. ATP [25] and FAD [26], as well as in activities of certain crucial enzymes, e.g. glutathione reductase [7] and glucose-6-phosphate dehydrogenase [9]. It also has been reported that the level of lipid peroxides in blood increases during riboflavin deficiency, and this increase has been positively correlated with cataractogenesis in rats [14].

Adriamycin®, a widely utilized anti-cancer agent, has substantial photosensitizing properties [17] which potentially may be detrimental to lens tissue, and our present study determined whether riboflavin deficiency compromises GSH concentration in lens tissue following systemic administration of this drug into rats.

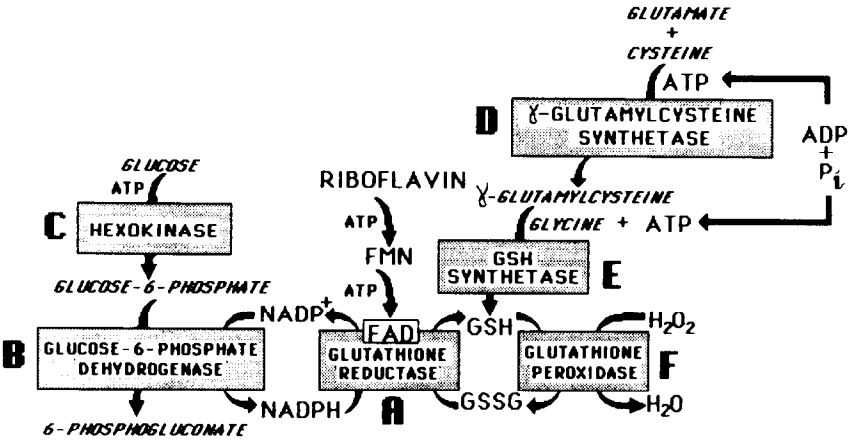

Fig. 2. Schema of glutathione metabolism in lens and proposed interference during riboflavin deficiency. The bold letters (A–F) adjacent to enzymes in the figure indicate the sites of interference which may occur in lens during riboflavin deficiency: (A) diminished activity of glutathione reductase due to decreased FAD levels (riboflavin deficiency) [7, 26]; (B) diminished activity of glucose-6-phosphate dehydrogenase due to decreased enzyme synthesis [9]; (C) diminished activity of hexokinase due to allosteric feedback regulation by increased glucose-6-phosphate concentration [26, 27]; (D/E) diminished activities of γ-glutamylcysteine synthetase and glutathione synthetase due to decreased availability of ATP (inhibition of anaerobic glycolysis, see point C) [9, 25]; and (F) diminished activity of glutathione peroxidase due to decreased availability of reduced glutathione [28].

Our study clearly shows that rats fed a diet replete in riboflavin did not exhibit diminished lens GSH concentration when treated for 3 days with Adriamycin®. Dietary riboflavin deficiency alone diminished GSH level to 74% of control value. Of primary concern is that Adriamycin® treatment of animals together with diminished intake of riboflavin depleted lens GSH concentration to approximately half of control values. In the time–course of this study, glucose-6-phosphate dehydrogenase activity in erythrocytes was not altered, suggesting that Adriamycin® treatment or deficiency of riboflavin either alone or in combination may not impose a significant generalized oxidative stress in the whole organism.

Since the amount of voluntary food intake of animals on the riboflavin-deficient diet is less than that required, control rats which are pair-fed to deficient animals subsequently receive inadequate dietary allowance and experience a marginal degree of riboflavin deficiency as indicated by the slight elevation (1.4 to 1.5) of their erythrocyte GRAC. As expected, glutathione reductase activity was diminished significantly and the activity coefficient was increased significantly in lens of riboflavin-deficient rats. Other investigators have reported previously that the activity of glucose-6-phosphate dehydrogenase is also decreased in lens during riboflavin deficiency [9]. Diminished activity of glucose-6-phosphate dehydrogenase can cause an increase in levels of glucose-6-phosphate, a feedback inhibitor of hexokinase, thus blocking glycolysis [27]. The end result of this may be decreased formation of ATP as well as NADPH, a coenzyme necessary for glutathione reductase activity. As illustrated in Fig. 2, riboflavin deficiency may deplete GSH concentration in lens by three different potential mechanisms: (a) inhibition of glutathione reductase activity through diminished availability of FAD, (b) a further decrease in glutathione reductase activity through decreased formation of NADPH, a second coenzyme of the enzyme, and (c) impaired *de novo* biosynthesis of GSH from amino acids due to a depletion of glycolysis-generated ATP. As a result of alterations in GSH regeneration, riboflavin deficiency has been reported to diminish the activity of glutathione peroxidase in lens [28]. Thus, increased generation of lenticular lipid peroxides observed during advanced degrees of riboflavin deficiency [28] may be due to a combination of decreased production of GSH and diminished activity of glutathione peroxidase.

Adriamycin® did not alter the activity or activity coefficient of glutathione reductase in these animals, suggesting that any synergistic effect with riboflavin deficiency on GSH depletion may not have resulted from diminished glutathione reductase activity. The significant decline in GSH concentration in lens of riboflavin-deficient animals by Adriamycin® may have resulted either from diminished biosynthesis or increased utilization of GSH since Adriamycin® has been reported to cause oxidative stress and lipid peroxidation [29].

In its entirety, our study shows that treatment with a photosensitizing drug, Adriamycin®, in the presence of riboflavin deficiency may be especially detrimental to the reducing capacity of lens tissue. Investigations are in progress to determine the mechanism responsible for the deleterious effect of Adriamycin® upon the decline in lenticular GSH concentration due to riboflavin deficiency. In addition, the consequences of diminished GSH concentration, the use of photosensitive drugs, and riboflavin deficiency in relation to structure and function of lens tissue should be explored.

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