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Hemolysis of Human Red Blood Cells by Riboflavin–Cu(II) System: Enhancement by Azide

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Abstract—Photoactivated riboflavin in the presence of Cu(II) generates reactive oxygen species (ROS) which can hemolyze human red blood cells (RBC). In the present work we examined the effect of sodium azide (NaN_3) on RBC in the presence of riboflavin and Cu(II). The addition of NaN_3 to the riboflavin–Cu(II) system enhanced K^+ loss and hemolysis. The extent of K^+ loss and hemolysis were time and concentration dependent. Bathocuproine, a Cu(I)-sequestering agent, inhibited the hemolysis completely. Among various free radical scavengers used to identify the major ROS involved in the reaction, thiourea was found to be the most effective scavenger. Thiourea caused almost 85% inhibition of hemolysis suggesting that $^{\cdot}OH$ is the major ROS involved in the reaction. Using spectral studies and other observations, we propose that when NaN_3 is added to the riboflavin–Cu(II) system, it inhibits the photodegradation of riboflavin resulting in increased $^{\cdot}OH$ generation. Also, the possibility of azide radical formation and its involvement in the reaction could not be ruled out.

Key words: riboflavin, copper, sodium azide, reactive oxygen species, RBC, hemolysis

Free radical-mediated oxidation of biological molecules, membranes, and tissues is believed to be related to a variety of pathological events like cancer and Alzheimer's disease [1, 2]. Oxidative modification of cellular constituents (including lipids, proteins, and nucleic acids) has been implicated in the etiology of different pathological conditions and in aging [3]. On the other hand, free radicals have been used with great success for the treatment of various forms of tumors. Attempts are constantly being made to extend this treatment modality to other clinical conditions. Moreover, free radicals also play an important role in various defense mechanisms against infections [4] and in tissue injury and inflammatory reactions [5].

Riboflavin in the presence of Cu(II) and light causes breakage of calf thymus DNA and super coiled plasmid DNA [6], degradation of bovine serum albumin (BSA) [7], and hemolysis of red blood cells (RBC) [8]. Several reports have shown that photoactivated riboflavin reacts with molecular oxygen to generate reactive oxygen species (ROS) [9, 10]. These ROS have been shown to damage rat lens [11], inactivate enzymes [12, 13], cause lipid peroxidation [14], increase protein cross-linking [15], and destroy bilirubin [16], uric acid [17], and amino acids [18, 19]. The damage caused by ROS is further exacerbated if the antioxidant enzymes themselves are damaged and inactivated by such events [20]. The administration of certain drugs commonly designated as oxidants stimulates the generation of ROS, which may overwhelm cellular protective mechanisms [21]. This is particularly so when the defense of the cell is already defective due to hereditary disorders. ROS are also intimately associated with the photodynamic effect of many drugs involved in cancer therapy [22].

The photodynamic action of riboflavin is of special interest because phototherapy is widely used in the treatment of neonatal jaundice [23] and a variety of solid neoplasms [24]. The principle of phototherapy is based on the selective uptake of a photosensitizing chemical in tumor tissues/cells followed by irradiation of the tumor with visible light. The treatment results in a cascade of oxidative events causing cell death. We have previously shown that irradiation of riboflavin with visible light causes RBC hemolysis in the presence of Cu(II) and this effect is mediated by ROS [8]. The present work shows the effect of sodium azide (NaN_3) on RBC hemolysis induced by the riboflavin–Cu(II) system. The unexpected enhancing effect of NaN_3 in this reaction is presented for the first time.

Abbreviations: ROS) reactive oxygen species; RBC) red blood cells.

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MATERIALS AND METHODS

Chemicals. Riboflavin and cupric (II) chloride were obtained from Sigma (USA). All other chemicals used were of the highest purity grade available commercially.

Preparation of RBC. RBC were prepared by centrifugation of fresh human blood, from different healthy volunteers, at 1500*g* for 10 min at room temperature. The cells were washed three times with five volumes of isotonic NaCl solution and then suspended in the desired buffer.

Measurement of K^+ loss. Packed RBC were suspended in 3 ml of buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and varying concentrations of riboflavin, Cu(II), and sodium azide to give 0.5% hematocrit. The reaction mixtures were incubated at room temperature in 800 lx cool fluorescent light. The distance between the light source and reaction tubes was 30 cm to exclude the side effect of heating. During the incubation period, the reaction tubes were gently inverted every 30 min to prevent cell sedimentation. After centrifugation at 1500*g* for 10 min the concentration of K^+ was measured in the supernatant using an EEL flame photometer (Evans Electroselenium Ltd., England). For reference of 100% K^+ loss, a sample of RBC was hemolyzed in distilled water and K^+ concentration was determined in the supernatant after centrifugation.

Measurement of hemolysis. The percent hemolysis following incubation of RBC with riboflavin, Cu(II), and NaN_3 was measured by reading the absorbance of the hemolysate at 415 nm as described by Yoshida et al. [25]. The reaction mixtures were incubated at room temperature in 800 lx cool fluorescent light. During the incubation period, the reaction tubes were gently inverted every 30 min to prevent cell sedimentation. For reference of 100% hemolysis, RBC were treated with distilled water. Wherever specified, varying amounts of free radical scavengers or bathocuproine were included in the reaction mixture (see figure legends for details).

RESULTS AND DISCUSSION

The incubation of human RBC in fluorescent light for 3 h with 50 μM riboflavin, 100 μM Cu(II), and increasing concentrations of NaN_3 resulted in progressive loss of intracellular K^+ and significant hemolysis (Fig. 1). There was 100% K^+ loss into the medium at 100 μM NaN_3. At this concentration of NaN_3, 60% hemolysis was observed. Maximum hemolysis (75%) was obtained at 150 μM NaN_3. Further increase in NaN_3 concentration did not result in any significant increase in hemolysis. The riboflavin–Cu(II) combination gave only 14% hemolysis and 30% K^+ loss. In light protected control samples, no loss of intracellular K^+ or hemolysis was observed even after prolonged incubation (data not shown). To investigate whether Cu(I) plays a role in hemolysis, bathocuproine, a Cu(I) sequestering agent, was included in the reaction medium. Complete inhibition of hemolysis was achieved at 60 μM of bathocuproine (Fig. 2). Several free radical scavengers were added to the

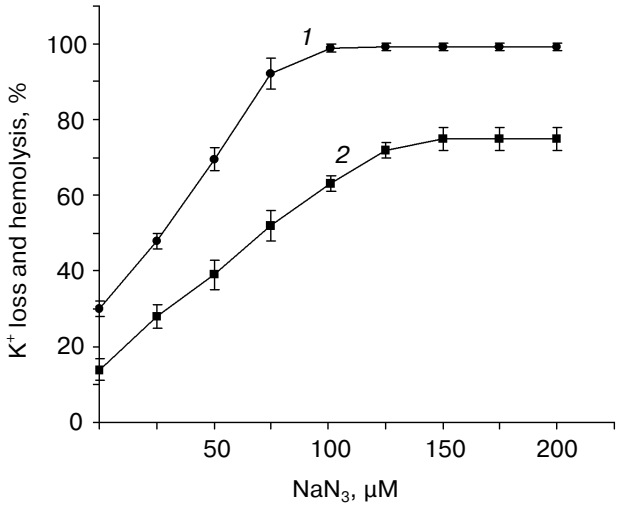

Fig. 1. Effect of increasing concentrations of NaN_3 on intracellular K^+ loss and hemolysis. RBC were incubated in 3 ml buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 50 μM riboflavin, 100 μM Cu(II), and 25-200 μM NaN_3 to give 0.5% hematocrit. The percentage of intracellular K^+ loss (*1*) and hemolysis (*2*) were determined after 3 h of incubation in fluorescent light. Values are mean ± SD ($n = 3$).

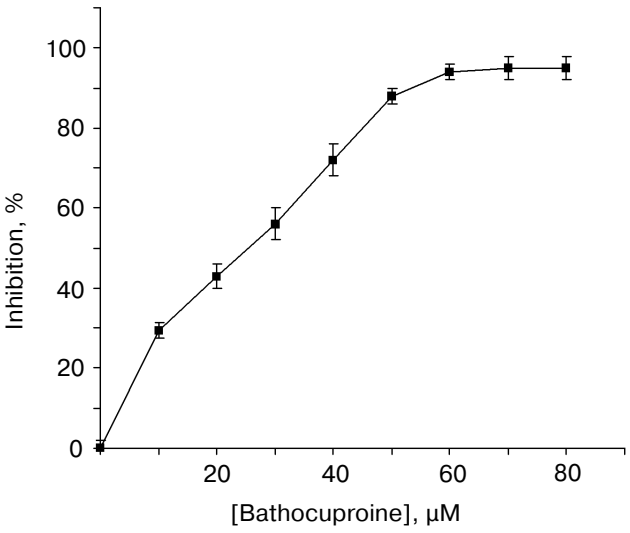

Fig. 2. Effect of bathocuproine on the hemolysis of RBC induced by riboflavin, Cu(II), and NaN_3. RBC were incubated with 50 μM riboflavin, 100 μM Cu(II), 150 μM NaN_3, and varying concentrations (10-80 μM) of bathocuproine. After 3 h of incubation in fluorescent light, percent hemolysis was determined and compared with controls lacking bathocuproine. Values are mean ± SD ($n = 3$).

reaction medium to identify the major ROS responsible for RBC hemolysis (Fig. 3). Potassium iodide, a scavenger of triplet oxygen, gave 38% inhibition of hemolysis. However, superoxide dismutase (a scavenger of superoxide anion), catalase (a scavenger of hydrogen peroxide), and β-carotene (a scavenger of both $^1O_2$ and nitric oxide) did not show any significant inhibition. In contrast, thiourea, a scavenger of hydroxyl radical (˙OH), resulted in 85% inhibition of hemolysis.

The spectral changes in riboflavin induced by exposure to 800 lx cool fluorescent light under different conditions were recorded between 300-500 nm (Fig. 4). Riboflavin exhibits a visible spectrum with a major peak of absorbance at 440 nm and a minor peak at 370 nm. Incubation of riboflavin for 30 min in fluorescent light caused a decrease in the absorption peak at 440 nm, suggesting photodegradation of riboflavin. The presence of Cu(II) inhibits the photodegradation and restores the 440 nm peak to some extent. These observations are in agreement with a previous report [10]. However, the addition of NaN_3 to the above reaction mixture significantly inhibited the photodegradation of riboflavin and restored the 440 nm peak of absorbance to about 85%.

Riboflavin (vitamin B_2) is the prosthetic group of several proteins and enzymes. When exposed to light, riboflavin absorbs energy and reacts with other molecules such as protonated substrate or molecular oxygen generating ROS [26]. Addition of Cu(II) to the riboflavin reaction inhibited the photodegradation of riboflavin. In this process, Cu(II) is reduced to Cu(I) and the later was found to be an essential intermediate in the generation of ˙OH [8]. Addition of NaN_3 to the riboflavin–Cu(II) reaction enhanced K^+ loss and hemolysis of human RBC compared to the reaction where NaN_3 was absent. The enhancing effect of NaN_3 is surprising and in contrast with the expected inhibition since NaN_3 is a known scavenger of $^1O_2$ [27]. In our reaction system, the enhancing effect of NaN_3 was consistent with time (data not shown) and concentration tested. To explore the possible mechanism involved, bathocuproine was included in the reaction containing NaN_3. The hemolysis was completely inhibited by bathocuproine suggesting that Cu(I) is an essential intermediate in the reaction. The role of ˙OH in the reaction was further confirmed by using different free radical scavengers. Thiourea caused almost 85% inhibition of hemolysis. The enhancing effect of NaN_3 can be explained from spectral studies of riboflavin. The addition of NaN_3 inhibits the photodegradation of riboflavin to a significant extent. This inhibition protects the riboflavin structure for a longer time, so more ˙OH can be generated and ultimately increase RBC hemolysis.

The possibility of azide radical ($N_3^{\cdot}$) formation from the reaction of ˙OH with azide anion (N_3^-) [1, 28] by the equation shown below could not be ruled out. Azide radical is difficult to detect directly [28] and is considered to be a strong one-electron oxidant capable of oxidizing thiols, other sulfur compounds, and phenols [29-31]:

$$N_3^- + {}^{\cdot}OH \rightarrow N_3^{\cdot} + OH^-.$$

However, more work is required to establish whether $N_3^{\cdot}$ is produced and involved in this system.

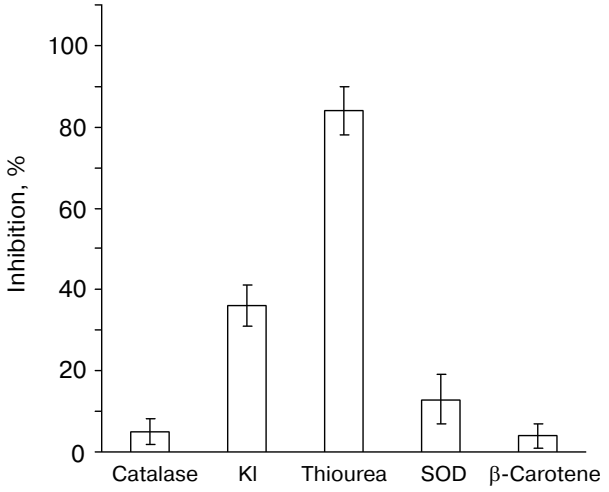

Fig. 3. Effect of various free radical scavengers on RBC hemolysis. RBC were incubated with 50 μM riboflavin, 100 μM Cu(II), 150 μM NaN_3, and 0.1 mM of either thiourea, potassium iodide, β-carotene, or 50 μg/ml of superoxide dismutase (SOD) or catalase. After incubation for 3 h in fluorescent light, percent hemolysis was determined. Values are mean ± SD (n = 3).

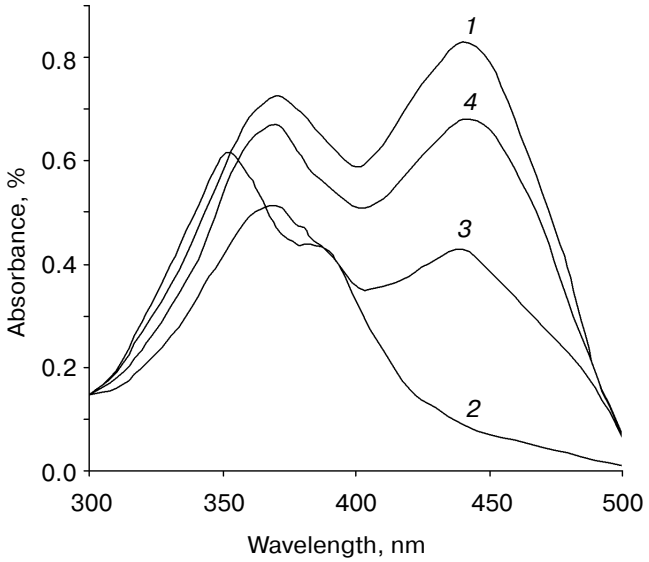

Fig. 4. Absorption spectra of riboflavin (50 μM): at zero time (*1*), after 30 min of incubation in cool fluorescent light (*2*), after 30 min of incubation in the presence of 100 μM Cu(II) (*3*), after 30 min of incubation in the presence of 100 μM Cu(II) and 150 μM NaN_3 (*4*).

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