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Hemolysis of human red blood cells by combination of riboflavin and aminophylline

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Abstract

The effect of aminophylline on human red blood cells (RBC) has been studied. Under in vitro condition, aminophylline alone does not hemolyse RBC. However, in the presence of riboflavin and visible light, aminophylline causes hemolysis of RBC. This hemolysis depends on the concentration of both riboflavin and aminophylline. Using different free radical scavengers we show that RBC hemolysis is caused by reactive oxygen species. Studies using bovine serum albumin show that riboflavin–aminophylline combination can also cause protein degradation in vitro.

Keywords: Riboflavin; Aminophylline; Oxygen radicals; RBC; Hemolysis

Introduction

There is a growing body of evidence that prooxidant species can contribute to lung injury in a variety of pulmonary diseases including asthma [1,2]. Enhanced production of free radicals has been reported particularly in asthmatic patients. This oxidant load has been found to correlate with the clinical severity of the disease and the entity of the airway obstructions [2,3]. Theophylline, and its more soluble form, aminophylline, are bronchodilator drugs commonly used for the treatment of asthma [4,5]. Both these drugs are administered

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intravenously during treatment. Both aminophylline and theophylline are xanthine compounds structurally related to uric acid [4], which has significant antioxidant property [1]. Recent studies have shown that theophylline, at therapeutically used concentrations, can decrease oxygen radical release from alveolar macrophages and mononuclear cells [6], suggesting that some indirect antioxidant mechanisms may also be operative in its therapeutic action [7]. On the other hand, theophylline, in combination with riboflavin, was also found to enhance bilirubin degradation in vitro. Therefore, a riboflavin–aminophylline combination was recommended for the effective treatment of jaundice in neonate [8].

We have recently shown that photoactivated riboflavin induces hemolysis of human red blood cells (RBC) in the presence of Cu(II) [9]. As aminophylline is administered intravenously, it was of interest to study whether the recommended riboflavin–aminophylline combination had any effect on human RBC. Unexpectedly, it was observed for the first time that a riboflavin–aminophylline combination causes hemolysis of fresh human RBC in the presence of visible light and in absence of Cu(II). However, neither riboflavin nor aminophylline was able to cause any hemolysis alone, either in the presence or absence of light.

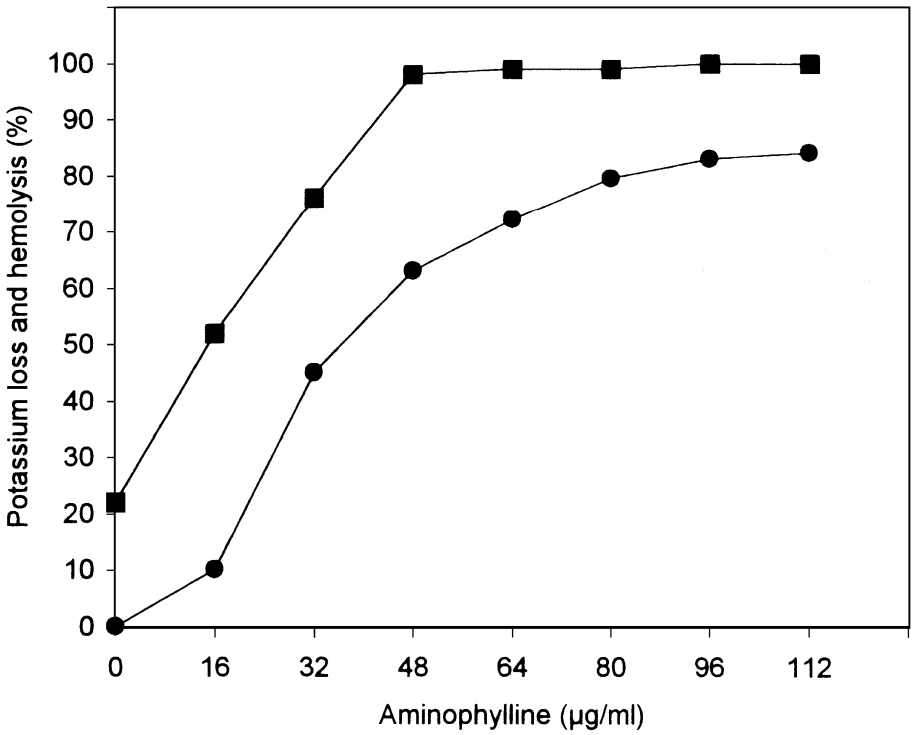

Fig. 1. Riboflavin–aminophylline induced K^+ loss and hemolysis of human RBC. Cells were incubated in 3 ml of buffer containing 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 50 μM riboflavin and 16–112 μg/ml aminophylline to give 0.5% hematocrit. The percent of K^+ loss (■) and hemolysis (●) was measured after 2 hours of incubation in visible light.

Methods

RBC were prepared by centrifugation of fresh human blood at 1500 xg for 10 minutes at room temperature. The cells were washed three times with five volumes of isotonic NaCl solution and suspended in the desired buffer.

Measurement of K^+ loss

Packed RBC were suspended in 3 ml of 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, varying amounts of riboflavin and aminophylline to give 0.5 % hematocrit. The reaction mixtures were incubated at room temperature in 800 lux of cool fluorescent light, and centrifuged at 1500 xg for 10 minutes.

The concentration of K^+ was measured in the supernatant using EEL flame photometry (Evans Electroselenium Halsted Essex, England). For reference of 100% intracellular K^+, a sample of RBC was hemolysed in distilled water and K^+concentration was determined in the supernatant after centrifugation as above.

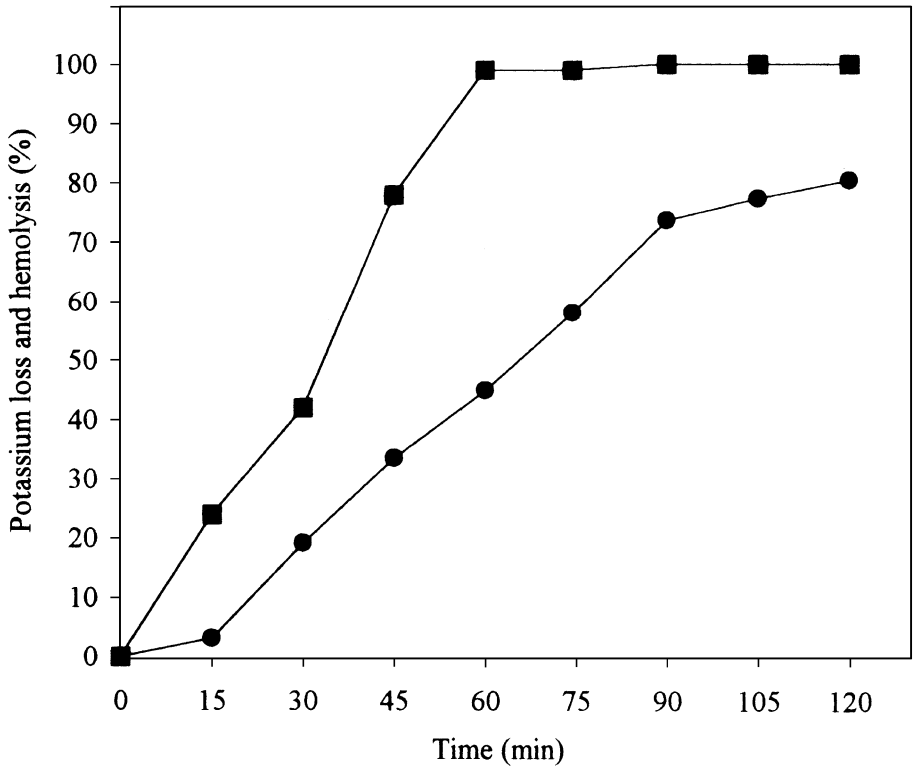

Fig. 2. Effect of increasing time of incubation on K^+ loss and hemolysis of human RBC. Cells were incubated with 50 μM riboflavin and 80 μg/ml aminophylline for different time intervals in visible light. (■) K^+ loss; (●) hemolysis.

Measurement of hemolysis

The percent hemolysis following incubation of RBC with riboflavin and aminophylline was measured by reading the absorbance of the hemolysate at 415 nm as described by Yoshida et al [10]. For reference, RBC were treated with distilled water and hemolysate read at 415 nm to obtain 100% hemolysis. All experiments were carried out in triplicate and the mean values are reported. Wherever specified, varying amounts of free radical scavengers were included in the reaction mixture (see figure legends for details).

Results

The incubation of human RBC in visible light in the presence of 50 μM riboflavin and increasing concentrations of aminophylline for 2 hours resulted in progressive loss of intracellular K^+ and significant hemolysis (Fig. 1). There was an initial increase in extracellular K^+, and at 48 μg/ml aminophylline 100% K^+ leaked out in the medium. At this stage 62% hemolysis was also observed, however further increase in aminophylline concentration did not

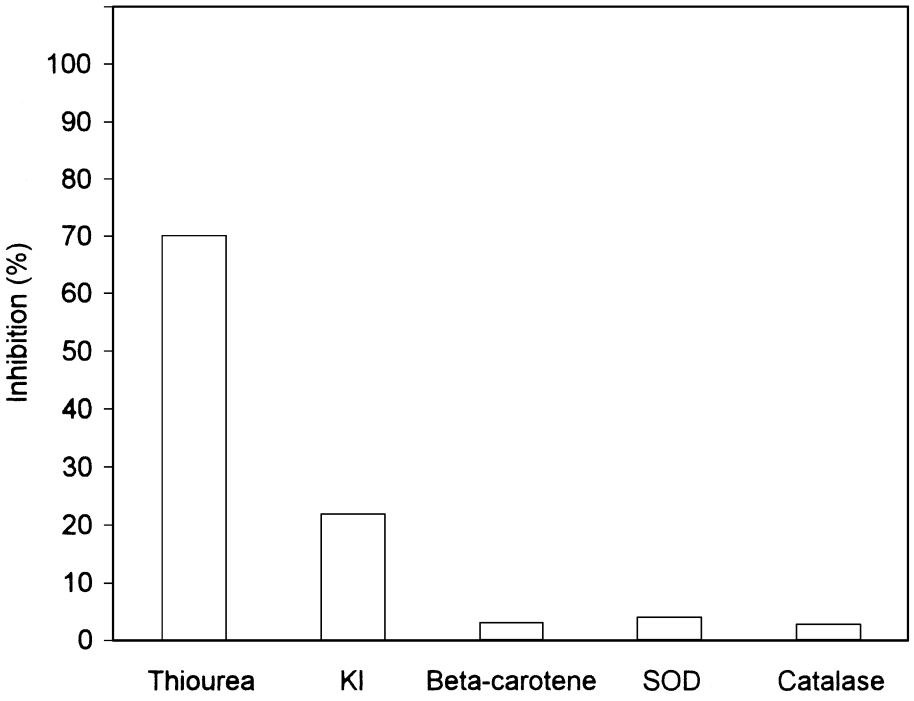

Fig. 3. Inhibition of human RBC hemolysis by various free radical scavengers. Cells were incubated with 50 μM riboflavin and 80 μg/ml aminophylline and 0.1 mM of either potassium iodide, thiourea, β-carotene or 20 μg/ml of SOD or catalase. The incubation was for 2 hours in fluorescent light.

result in further increase in hemolysis. No hemolysis was observed even after prolonged incubation time in the absence of aminophylline.

The percent K^+ loss and hemolysis in the presence of 50 μM riboflavin and 80 μg/ml aminophylline were determined as a function of time (Fig. 2). After 1 hour of incubation under visible light 100% of K^+ was leaked out in the medium with 45% hemolysis. Increasing the time of incubation to 2 hours resulted in 80% hemolysis. In light-protected control samples, no loss of intracellular K^+ or hemolysis was observed even in the presence of both riboflavin and aminophylline (data not shown), suggesting that the reaction is light mediated.

Since photoilluminated riboflavin mediates various damaging reactions via reactive oxygen species (ROS), several free radical scavengers were included in the reaction to find out the major ROS responsible for RBC hemolysis. Potassium iodide, a scavenger of triplet oxygen ($^3O_2$) showed 22% inhibition (Fig. 3). Thiourea, a scavenger of hydroxyl radical (OH), showed 70% inhibition of RBC hemolysis. Other scavengers like superoxide dismutase, β-carotene and catalase did not show significant inhibition.

The spectral changes in riboflavin induced by light under different conditions were recorded (Fig. 4). Riboflavin exhibits a visible spectrum with a major peak of absorption

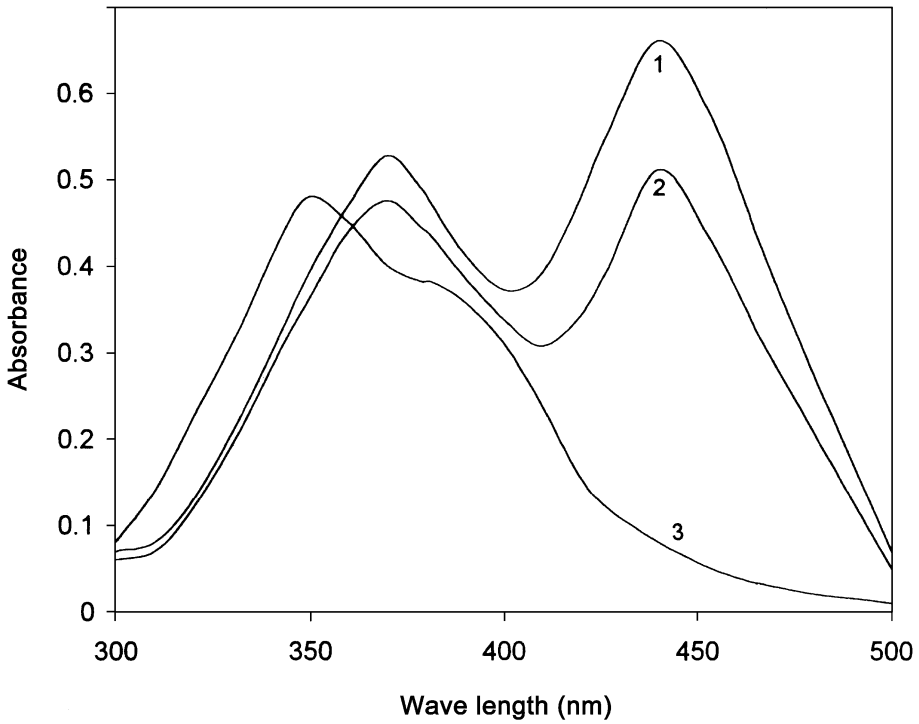

Fig. 4. Absorption spectra of riboflavin under different conditions. 50 μM riboflavin alone at zero time (1), riboflavin alone after 1 hour of incubation under visible light (3), riboflavin with aminophylline and 0.1 mM thiourea after 1 hour of incubation (2). Inclusion of aminophylline up to 100 μg/ml has no effect of the riboflavin spectra before and after incubation in light.

at 440 nm and a minor peak at 370 nm. Incubation of riboflavin under fluorescent light for 30 minutes caused a decrease in the absorption peak at 440 nm, suggesting photodegradation of riboflavin. The presence of aminophylline did not have any effect on the light mediated disappearance of the peak at 440 nm. Interestingly, the addition of thiourea to the riboflavin–aminophylline mixture inhibited the photodegradation of riboflavin and restored the peak at 440 nm to a significant extent, thiourea also inhibits RBC hemolysis significantly as shown above (Fig. 3).

To determine whether aminophylline undergoes any structural change or exhibits binding to riboflavin during irradiation, the absorption spectrum of aminophylline was recorded between 240–300 nm (Fig. 5). Aminophylline exhibits a UV spectrum with a peak at 270 nm. Irradiation of aminophylline with fluorescent light for more than 2 hours did not cause any change in the absorption peak at 270 nm (data not shown). Addition of 50 μM riboflavin to the reaction caused a decrease in the peak within 10 minutes of irradiation (data not shown). Moreover, the absorption peak of aminophylline completely vanished in the presence of riboflavin after 30 minutes of incubation in fluorescent light. However, the addition of sodium

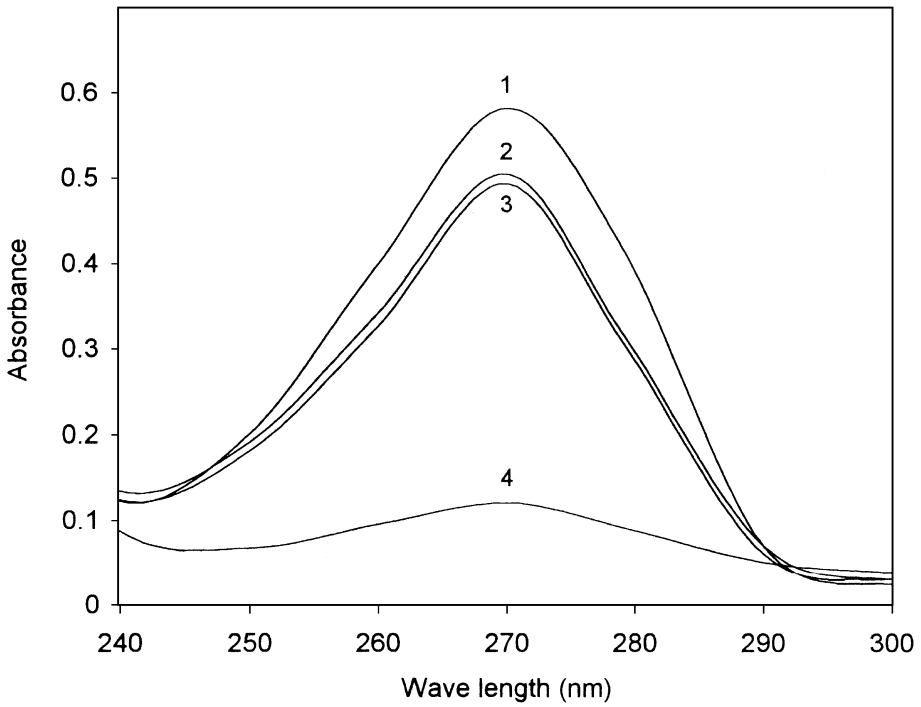

Fig. 5. The absorption spectra of aminophylline with riboflavin under different conditions. The spectra of 80 μg/ml aminophylline in the presence of 50 μM riboflavin was recorded between 240–300 nm after irradiation with fluorescent light. Aminophylline alone at zero time (1), aminophylline and riboflavin with sodium azide (2) or potassium iodide (3) after 30 minutes of incubation, aminophylline with riboflavin after 30 minutes of incubation (4).

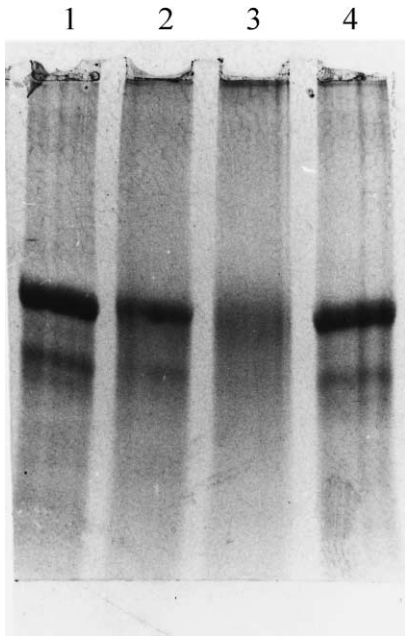

Fig. 6. Degradation of BSA by photoilluminated riboflavin and aminophylline. 15 μg/ml BSA alone (lane 1), 15 μg/ml BSA and 50 μM riboflavin (lane 2), 15 μg/ml BSA, 50 μM riboflavin and 80 μg/ml aminophylline (lane 3), 15 μg/ml BSA, 50 μM riboflavin, 80 μg/ml aminophylline and 0.1 mM thiourea (lane 4).

azide or potassium iodide to the medium inhibited this photodegradation and almost restored the peak at 270 nm to normal.

We have studied the effect of riboflavin–aminophylline combination using BSA as the target molecule. This was done to find out whether the effect of this combination is restricted to RBC damage or it can function in vitro on other systems also. Riboflavin alone caused only a slight change in BSA (Fig. 6, lane 2), while in the presence of aminophylline, BSA is almost completely degraded to small fragments (lane 3) and the effect is non specific as shown by the smearing of the major band. The presence of thiourea in the reaction significantly inhibited BSA degradation (lane 4) showing the involvement of $OH^{\bullet}$ as the major ROS. The irradiation of BSA in the presence of aminophylline alone had no effect (data not shown).

Discussion

Riboflavin or vitamin B2 is the prosthetic group of several proteins and enzymes where it is reversibly reduced by hydrogen atoms. When exposed to light, riboflavin absorbs energy and reacts via its singlet and triplet excited states with other molecules such as protonated substrate or molecular oxygen to generate ROS [11]. These ROS are well known for their damaging effect on several biological molecules [12,13]. ROS also play a role in the etiology

of lung pathologies such as emphysema, adult respiratory distress syndrome, and asthma [14]. It has been reported that riboflavin can induce hemolysis of rat RBC through free radical mediated reaction, if serum is included in the reaction [15]. Furthermore, we have previously shown that riboflavin, when illuminated in the presence of Cu(II) but complete absence of serum, caused significant hemolysis of human RBC, due to the generation of variety of ROS, including $OH^{\bullet}$ [9]. However in the absence of Cu(II) and serum, riboflavin only caused K^{+} leakage [9,15].

In the present work we have studied the effect of photoactivated riboflavin on RBC in the presence of aminophylline. As shown here, aminophylline caused the riboflavin-sensitized photohemolysis of human RBC in vitro, but is without any effect in the absence of riboflavin even after prolonged incubation. The reaction was found to be light mediated since no hemolysis occurred in light protected sample. Significantly, the hemolysis reaction did not require presence of Cu(II). Based on the spectral studies of aminophylline and riboflavin as well as on our previous findings [12,16], we suggest that riboflavin upon photoexcitation

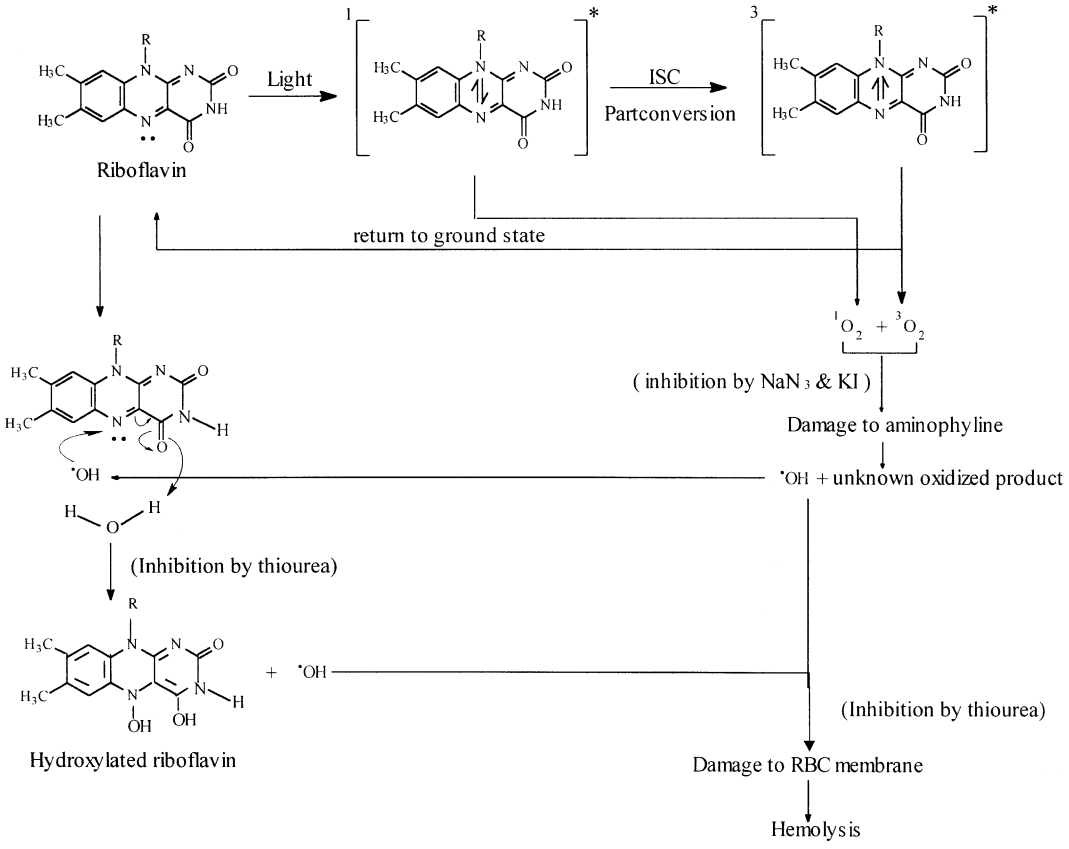

Fig. 7. A scheme for the photodegradation of riboflavin–aminophylline and RBC damage in the presence of fluorescent light (partially drawn from our previous paper (16)). ISC = Intersystem conversion; ^{1}[= singlet state; ^{3}[= triplet state; * = nπ*; R = CHOH–CHOH–CHOH–CHOH–CH_2OH.

generates the singlet state, which through intersystem conversion partially gives rise to triplet state. These singlet and triplet states of riboflavin by transferring energy to molecular oxygen give rise to $^1O_2$ and $^3O_2$ species, which are very reactive and hence attack aminophylline. Aminophylline is converted to some unknown oxidized product and releases $OH^\bullet$, therefore, there is decrease in the absorption at 270 nm (Fig. 5). These $OH^\bullet$ damage RBC membrane causing hemolysis. They also attack riboflavin in the ground state and convert it to hydroxylated riboflavin and loss of conjugation leading to the decrease of the absorption peak at 440 nm (Fig. 4). The scheme given in Fig. 7, is supported by the observation that hemolysis of RBC is substantially inhibited by $OH^\bullet$ scavengers, while aminophylline peak of 270 nm is restored to a significant extent when sodium azide or potassium iodide are included in the incubation mixture. This reaction seems to be different from our previous study [9], where a divalent metal ion like Cu(II) is essentially required. The major ROS generated in the process is probably $OH^\bullet$ as only thiourea gave about 70% inhibition, while potassium iodide gave 22%. Similar effect of riboflavin–aminophylline combination is also seen in vitro when BSA is used as a target molecule and this effect was inhibited by thiourea as in case of RBC.

Conclusion

Theophylline is widely used for the treatment of idiopathic respiratory distress syndrome and apnea attacks in the newborn [11]. Serum levels of 8-12 μg/ml theophylline are assumed to be therapeutically effective. However, in individuals in whom the theophylline plasma clearance is reduced for any reason, even a single intravenous dose may result in increased serum levels and potential toxicity. As theophylline is also distributed through breast milk, intravenous administration of theophylline to a post pregnancy woman may cause irritability, or other signs of toxicity in nursing infants, especially, if the infant is undergoing photo-therapy for treatment of neonatal jaundice.

However the effect of aminophylline in combination with riboflavin in vivo remains to be examined. Therefore, further studies are necessary to elucidate these points using an in vivo system.

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