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Photodynamic inactivation of trypsin by the aminophylline-riboflavin system: Involvement of hydroxyl radical

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: Riboflavin finds ubiquitous occurrence in plants and animals and functions as a coenzyme participating in various oxidation-reduction reactions during the course of metabolism. Photosensitized riboflavin generates reactive oxygen species (ROS). Aminophylline is an antiasthmatic drug and a known phosphodiesterase inhibitor. In this study we examined the effect of photoilluminated riboflavin on aminophylline using trypsin as the target molecule.

Material/Methods: The possible loss of trypsin activity due to autolysis was assayed after incubation in fluorescent light. Changes in trypsin activity caused by photoilluminated riboflavin alone and with aminophylline were monitored as functions of concentration and time. These effects were also analyzed by SDS-PAGE to visualize protein degradation. Spectra of riboflavin, alone and with aminophylline, under different conditions were taken to monitor the structural changes for elucidating the possible reaction mechanism involved. Free radical scavengers were also included in some experiments.

Results: Aminophylline alone is not known to posses any photosensitizing characteristics. However, in the presence of riboflavin and fluorescent light, aminophylline caused inactivation and fragmentation of trypsin. This fragmentation was found to be concentration dependent and was mediated by ROS. In all cases, thiourea, a scavenger of hydroxyl radicals, was most effective in scavenging the damaging effect of the riboflavin-aminophylline combination.

Conclusions: Based on our results we suggest that photoilluminated riboflavin generates the singlet and triplet excited states that, upon energy transfer, generate $^1O_2^{\cdot}$ and $^3O_2^{\cdot}$ oxygen. These activated oxygen species probably attack aminophylline leading to its oxidation, generating hydroxyl radicals which presumably cause inactivation and fragmentation of trypsin.

key words: **Riboflavin • Aminophylline • Trypsin • Reactive Oxygen Species**

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BACKGROUND

Riboflavin (6, 7 dimethyl-9-D-1ribityl-iso-alloxazine) is known to possess a photosensitization characteristic [1]. When exposed to UV radiation from sunlight, riboflavin induces photo-damage to the skin [2]. Photoexcited riboflavin acts as a light-induced electron donor [3]. It reacts via its singlet and triplet excited states with molecular oxygen and generates ROS [4], such as the short-lived superoxide anion, singlet oxygen, and the riboflavin radical [5]. A substantial amount of hydrogen peroxide is also produced [6], which has a longer half-life and penetrates cell membranes freely. These species exert a collective damaging effect on several biological molecules [7,8] caused K^+ leakage and, in the presence of Cu (II), induced hemolysis of red blood cells [9] and damage lens [10], leading to oxidative modification of cellular constituents including lipids, proteins, and nucleic acids. Thus, these ROS have been implicated in the etiology of various pathological conditions and in aging [11,12]. Recent reports suggested a role of ROS and decreased SOD activity in an autoimmune condition called alopecia areata [13]. It has also been recently suggested, however, that riboflavin itself can play the role of an antioxidant by stabilizing the NO level in the organism under conditions of increased superoxide anion generation and/or decreased SOD activity, thereby playing a role in the modulation of toxic and signaling pathways of NO [14]. In addition, riboflavin photosensitization is known to produce a reducing species, the radical anion of lipoic acid, a powerful natural antioxidant which exerts significant antioxidant activity *in vivo* by deactivation of reactive oxygen and nitrogen species (ROS and RNS) [15].

Aminophylline is the more soluble analogue of theophylline. It is an inhibitor of phosphodiesterase [16]. The administration of aminophylline is known to increase the cAMP/cGMP ratio. These cyclic nucleotides have been shown to regulate fibroblast proliferation and collagen production like, the increase in total lung collagen normally seen after injury in pulmonary fibrosis, while having no effect on collagen levels in the undamaged lung [17].

Dent et al. [18] showed that theophylline inhibits the respiratory burst of alveolar macrophages by elevation of cAMP, while others [19] reported a similar cAMP-dependent negative effect of this agent on neutrophil activation. Therapeutically relevant concentrations of theophylline increased the level of cAMP and caused a dose-related inhibition of superoxide radical generation by human neutrophils, suggesting that some indirect antioxidant mechanisms may also be operative in its therapeutic action [20]. Since neutrophil-derived reactive oxidants cause damage to the respiratory epithelium in a number of respiratory diseases, including allergic inflammation induced by ROS in asthma [21] and chronic obstructive airway disease, this accounts, at least in part, for the beneficial anti-inflammatory role of theophylline seen in these respiratory disorders [22]. Theophylline is also widely used for the treatment of idiopathic respiratory distress syndrome and apnea attacks in newborns [4]. Serum levels of 8–12 μg/ml theophylline are assumed to be therapeutically effective. Theophylline can inhibit cell infiltration and serum protein leakage in asthma [23].

Theophylline in combination with riboflavin was found to enhance bilirubin degradation *in vitro*, and the crossreactivity of photoactivated riboflavin with other drugs, such as timolol and pindolol, has already been studied [24]. Also, a riboflavin-aminophylline combination was recommended for the effective treatment of jaundice in neonates [25]. With the possible antioxidant effect of aminophylline known, it was of interest to study riboflavin in combination with aminophylline.

MATERIAL AND METHODS

Material

Trypsin, (EC 3.4.21.4) was obtained from SRL, India, thiourea from Qualigens Fine Chemicals, India, while aminophylline, riboflavin, bovine serum albumin, catalase, sodium azide, and superoxide dismutase were purchased from Sigma Chemical Co, USA. All other chemicals used were of the highest purity grade available.

Assay of trypsin

Trypsin was assayed by the method of Kunitz et al. [26] using casein as the substrate. The reaction mixture in a final volume of 2 ml contained 10 mg casein, 10 mM phosphate buffer, pH 7.4, and increasing concentrations of trypsin (0.5–15 μg). After incubating at 37°C for 30 minutes, the reaction was terminated by the addition of 1 ml of 10% TCA. The samples were then centrifuged at 2500 rpm for 10 minutes to remove the undigested protein as precipitate, and the supernatant was used for determining the acid-soluble peptides using the method of Lowry et al. [27]. In a parallel experiment, trypsin alone was incubated under 800 lux of cool fluorescent light for 30 minutes prior to assay (to check for possible autolysis).

Inactivation of trypsin by photoilluminated riboflavin alone and with aminophylline

The reaction mixture in a final volume of 2 ml contained 10 mg casein, 10 mM phosphate buffer, pH 7.4, and 15 μg of trypsin preincubated under 800 lux of cool fluorescent light for 30 minutes with increasing concentrations of riboflavin (25–200 μM). Reactions were terminated by adding 1 ml of 10% TCA. Undigested protein was removed by centrifugation at 2500 rpm for 10 minutes and supernatant was used for estimation of acid-soluble peptides. In another experiment, trypsin was incubated under 800 lux of cool fluorescent light with 50 μM riboflavin and increasing concentrations of aminophylline (25–200 μg/ml).

SDS-polyacrylamide gel electrophoresis

Treated trypsin was resolved by 15% SDS-PAGE using a discontinuous buffer system [28] under reducing conditions. Reactions containing 50 μg of trypsin were incubated with riboflavin alone and riboflavin with aminophylline under 800 lux of cool fluorescent light for different time intervals. Various scavengers were also used in some experiments. The samples containing 30 μg of protein were electrophoresed for 2–3 hours at 80 V and the gels were visualized by staining with silver nitrate [29].

Silver staining

After electrophoresis, gels were immersed in a mixture of 30 ml of 50% acetone, 0.75 ml of 50% TCA, and 12.5 μl of

37% formaldehyde for 10 minutes with constant shaking for fixation, then they were washed three times with distilled water. Gels were again soaked in 30 ml of 50% acetone for 10 minutes. After this, they were treated with a solution containing 30 ml of distilled water and 50 µl of 10% $Na_2S_2O_3$. Next they were washed with distilled water. They were then soaked in a solution containing 0.4 ml 20% silver nitrate, 0.3 ml of 37% formaldehyde, and 30 ml of distilled water for 8 minutes. After washing with distilled water, the gels were transferred to a solution containing 0.02 g/ml of sodium carbonate, 12.5 µl of 37% formaldehyde, and 12.5 µl of 10% $Na_2S_2O_3$ to make them alkaline. After 10–20 seconds they were suspended in 1% glacial acetic acid for 1 minute to stop the reaction. Then they were finally washed with distilled water since the bands, if any, were visible by now.

The absorption spectra of riboflavin and aminophylline

The absorption spectra of riboflavin was recorded using a Cintra 5 UV-Visible Spectrometer (GBC Scientific Equipments) before and after incubation under 800 lux of cool fluorescent light at various time intervals (0, 15, 30, 45, 60, and 90 minutes). The reaction mixture in final volume of 3 ml contained 50 µM riboflavin in 10 mM phosphate buffer, pH 7.4. The sample was read against a phosphate buffer blank. The decrease in absorbance at 370 nm and 440 nm peaks after incubation in fluorescent light, is indicative of progressive photodegradation of riboflavin. The samples for recording aminophylline spectra under different conditions in a final volume of 3 ml contained 75 µg/ml aminophylline alone (a), with 50 µM riboflavin (c), and with 0.1 mM thiourea (d) in 10 mM phophate buffer, pH 7.4. The samples containing aminophylline alone, with riboflavin, and with thiourea were read between 240 nm to 300 nm using a Cintra 5 UV-Visible Spectrometer at time zero and after 1 hour of incubation under 800 lux of cool fluorescent light.

The results presented in this study are the mean of three independent experiments until and otherwise stated.

RESULTS

The present study focuses on the damaging effects of photoilluminated riboflavin alone and of its combination with aminophylline (an antiasthmatic drug) on trypsin as the target molecule.

Absorption spectra of riboflavin

The spectral changes in riboflavin induced by incubation in cool fluorescent light were recorded at different time intervals (Figure 1). Riboflavin exhibits a visible spectrum with a major peak of absorbance at 440 nm and a minor peak at 370 nm. Incubation of riboflavin under 800 lux of cool fluorescent light for 90 minutes caused the disappearance of the absorption peak at 440 nm, suggesting photodegradation of riboflavin.

Absorbance spectra of aminophylline in the presence of riboflavin

To determine whether aminophylline undergoes any structural change or exhibits binding to riboflavin during photoillumination, the absorption spectrum of aminophylline was recorded between 240–300 nm (Figure 2). Aminophylline

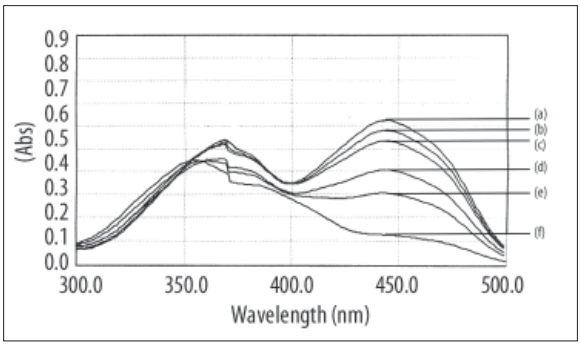

Figure 1. Absorption spectra of riboflavin at different time intervals. (a) 50 µM riboflavin at zero time, (b,c,d,e,f) riboflavin after 15 minutes, 30 minutes, 45 minutes, 1 hour, 1.5 hour of incubation in fluorescent light respectively.

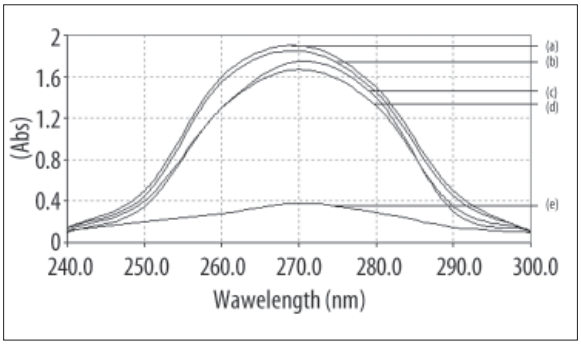

Figure 2. Absorption spectra of aminophylline with riboflavin under different conditions. The spectra of 75 µg/ml aminophylline in the presence of 50 µM riboflavin was recorded between 240—300 nm after irradiation with fluorescent light. (a) aminophylline alone at zero time, (b) aminophylline alone after 1 hour of incubation, (c) aminophylline with riboflavin at zero time, (d) aminophylline and riboflavin with thiourea after 1 hour of incubation, (e) aminophylline and riboflavin after 1 hour of incubation.

exhibits a UV-spectrum with a peak at 270 nm. Illumination of aminophylline in cool fluorescent light for more than one hour did not cause any change in the absorption peak at 270 nm. However, addition of 50 µM riboflavin to it caused a decrease in the peak within 30 minutes of illumination in cool fluorescent light. The absorption peak of aminophylline completely disappeared in the presence of riboflavin after 60 minutes of incubation in cool fluorescent light, indicating degradation of aminophylline caused by photoactivated riboflavin. The 270 nm peak of aminophylline was almost completely restored upon the addition of 0.1 mM thiourea, a known scavenger of hydroxyl radical, up to 60 minutes of incubation, indicating that hydroxyl radicals are produced during the photodegradation of aminophylline mediated by riboflavin. Other scavengers of ROS, such as catalase, SOD, potassium iodide, and sodium azide, had no significant effect on the aminophylline degradation (data not shown).

Inactivation of trypsin by riboflavin alone and riboflavin with aminophylline

When the assay of trypsin was performed by the method of Kunitz et al. [26] using casein as a substrate, the activity of

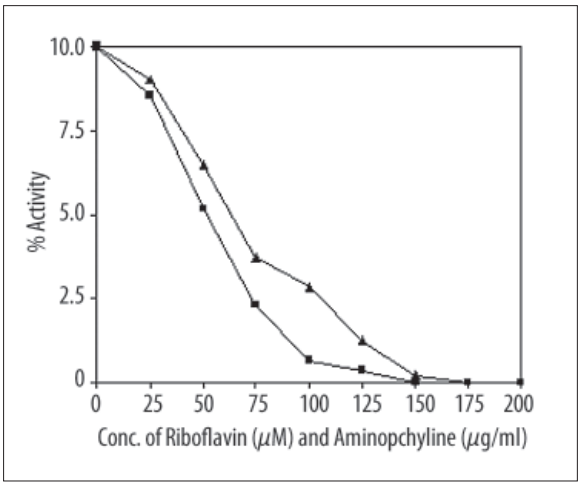

Figure 3. Inactivation of trypsin by increasing concentration of riboflavin alone as well as riboflavin and aminophylline. 15 µg of trypsin per sample was incubated with different concentrations of riboflavin (20–200 µM) for 30 minutes in fluorescent light (▲). In a parallel experiment 15 µg of trypsin per sample was incubated with 50 µM riboflavin and increasing concentrations of aminophylline (25–200 µg/ml) for 30 minutes in fluorescent light (■).

trypsin was found to increase as a function of enzyme concentration. In a parallel experiment, the assay was also performed with trypsin alone after it was incubated at room temperature for half an hour to test for possible autolysis. The decrease in activity was almost 20% in these samples, which was probably due to autolysis (data not shown).

The decrease in the activity of trypsin was monitored after incubating it in cool fluorescent light for half an hour with increasing riboflavin concentrations alone and with aminophylline. An increase in the concentration of riboflavin led to a parallel decrease in the activity of trypsin. Complete loss of activity was observed at 175 µM riboflavin. With 50 µM riboflavin and increasing aminophylline concentrations, complete loss of activity was observed at 150 µg/ml aminophylline (Figure 3).

The decrease in the activity of trypsin by riboflavin alone and by riboflavin-aminophylline was monitored as a function of time of illumination. Samples containing 50 µM riboflavin alone and those containing 50 µM riboflavin with 75 µg/ml aminophylline were photoilluminated for different time intervals. After 10 minutes of incubation in cool fluorescent light, there was very little decrease in the activity of trypsin with riboflavin alone. However, when aminophylline was included in the reaction, the inhibition of trypsin activity was around 60 percent. After 30 minutes of incubation in light, the enzyme was almost completely inactivated (data not shown).

Degradation of trypsin by the riboflavin-aminophylline system

After treatment of trypsin with riboflavin alone and with riboflavin–aminophylline combinations in cool fluorescent light, trypsin was applied on SDS-PAGE. It was found to be degraded significantly when incubated with riboflavin alone (Figure 4A); the degradation of trypsin was visible at 50 µM riboflavin and it was completed at 150 µM riboflavin, with the major band of trypsin has almost completely disappeared in two hours of incubation (lane e). In another experiment (Figure 4B), 50 µg trypsin was photoilluminated with 50 µM riboflavin and increasing concentrations of aminophylline (25–150 µg/ml). The major band of trypsin completely disappeared at 150 µg/ml aminophylline in the presence of riboflavin (lane f) when incubated for 30 minutes, in comparison with riboflavin alone, where incubation was for 2 hours, indicating enhanced degradation.

Effect of different free radical scavengers on trypsin degradation by the riboflavin-aminophylline system

Samples containing 50 µg trypsin and 50 µM riboflavin were photoilluminated for two hours in the presence of different free radical scavengers (Figure 5A). Trypsin degradation was slightly inhibited by potassium iodide, a scavenger of triplet oxygen, and thiourea, a scavenger of hydroxyl radicals. However, it was almost completely inhibited by SOD, which is a scavenger of the superoxide anion, as evident in Figure 5A, lane f. It is well reported that riboflavin upon photoillumination generates the superoxide anion. In a parallel experiment (Figure 5B), samples containing 50 µg trypsin, 50 µM riboflavin and 75 µg/ml aminophylline were photoilluminated for one hour. The degradation was completely inhibited by thiourea (lane g), suggesting hydroxyl radicals are the major ROS involved in the degradation of trypsin, when riboflavin-aminophylline were used in combination.

Discussion

As a coenzyme, riboflavin is responsible for the growth and development of the fetus and the maintenance of the mucosal epithelium and eye tissue [1]. UV light-activated riboflavin has been shown to oxidize guanine in nucleic acids *in vitro* [30]. Due to its planar structure, its ability to intercalate within DNA strands, and its photosensitizing property, riboflavin can prevent genome replication, thereby inactivating pathogens found in blood products. Riboflavin, as an endogenous photosensitizer, has an extremely good safety profile and can inactivate high levels of a broad range of viruses and bacteria in platelet concentrates, fresh frozen plasma, and red blood cells, preserving the activity and functionality of the components while reducing the risks of transfusion-transmitted disease. The photodegradation of riboflavin in the body is clearly shown by the decrease in its concentration in neonates who are treated with intense visible light to break down circulating bilirubin, which their immature livers cannot yet handle [31].

Aminophylline and theophylline are both intravenously administered bronchodilator drugs commonly used for the treatment of asthma [32]. These compounds are methylxanthines, structurally related to uric acid [33], which has a significant antioxidant property [34]. These drugs have been shown to possess anti-inflammatory [22] and immunomodulatory effects [35]. The mechanism of anti-inflammatory action has not been clearly elucidated.

In the present work we studied the effect of photoilluminated riboflavin in the presence of aminophylline using trypsin

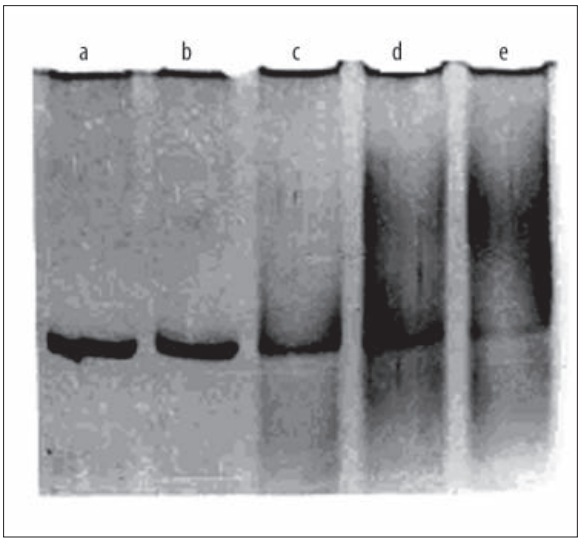

Figure 4A. Modification of trypsin with increasing concentration of riboflavin. 50 µg of trypsin per reaction sample was incubated in fluorescent light for 2 hours with increasing concentration of riboflavin; (lane a) trypsin alone, (lane b) trypsin with 25 µM riboflavin, (lane c) trypsin with 50 µM riboflavin, (lane d) trypsin with 100 µM riboflavin, (lane e) trypsin with 150 µM riboflavin.

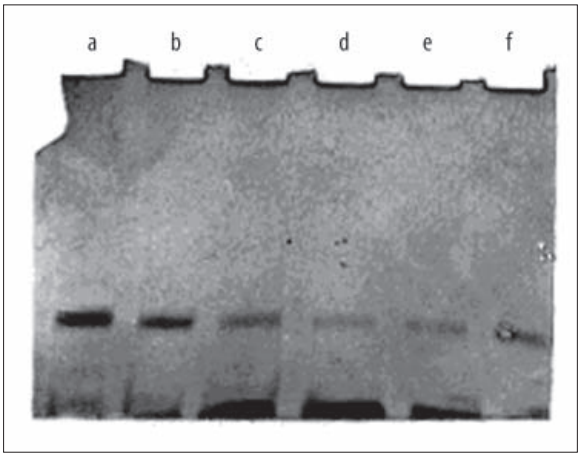

Figure 4B. Modification of trypsin with riboflavin and increasing concentration of aminophylline. 50 µg of trypsin per reaction sample was incubated in fluorescent light for 30 minutes with 50 µM riboflavin and increasing concentration of aminophylline; (lane a) trypsin alone, (lane b) trypsin with 50 µM riboflavin, (lane c) trypsin with 50 µM riboflavin and 25 µg/ml aminophylline, (lane d) trypsin with 50 µM riboflavin and 75 µg/ml aminophylline, (lane e) trypsin with 50 µM riboflavin and 125 µg/ml aminophylline, (lane f) trypsin with 50 µM riboflavin and 150 µg/ml aminophylline.

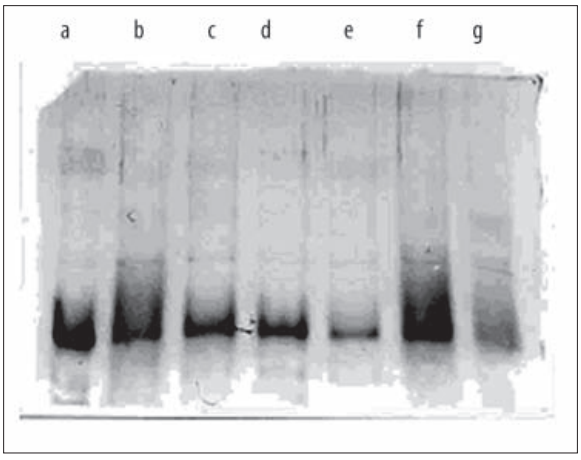

Figure 5A. Effect of riboflavin on trypsin in the presence of different free radical scavengers. 50 µg of trypsin per reaction sample was incubated in fluorescent light for 2 hours with different scavengers. (lane a) trypsin alone, (lane b) trypsin with 50 µM riboflavin, (lane c) trypsin with 50 µM riboflavin and 0.1 mM thiourea, (lane d) trypsin with 50 µM riboflavin and 20 µg/ml catalase, (lane e) trypsin with 50 µM riboflavin and 0.1 mM KI, (lane f) trypsin with 50 µM riboflavin and 20 µg/ml SOD, (lane g) trypsin with 50 µM riboflavin and 150 µM NaN_3.

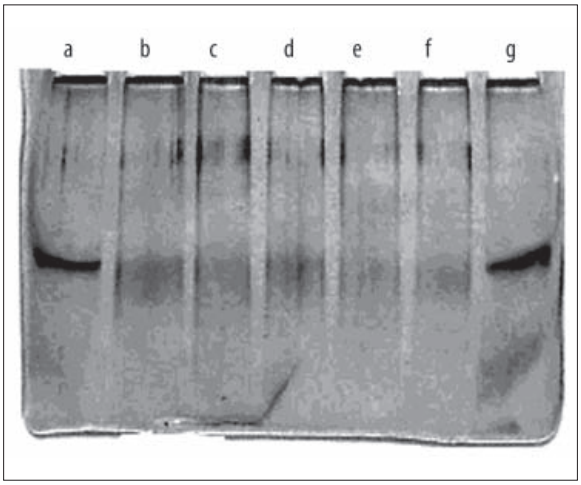

Figure 5B. Effect of riboflavin and aminophylline on trypsin in the presence of different free radical scavengers. 50 µg of trypsin, 50 µM riboflavin and 75 µg/ml aminophylline per reaction sample was incubated in fluorescent light for 1 hour with different scavengers; (lane a) trypsin alone, (lane b) trypsin with 50 µM riboflavin and 75 µg/ml aminophylline, (lane c) trypsin with 50 µM riboflavin, 75 µg/ml aminophylline and 20 µg/ml SOD, (lane d) trypsin with 50 µM riboflavin, 75 µg/ml aminophylline and 20 µg/ml catalase, (lane e) trypsin with 50 µM riboflavin, 75 µg/ml aminophylline and 150 µM NaN_3, (lane f) trypsin with 50 µM riboflavin, 75 µg/ml aminophylline and 0.1 mM KI, (lane g) trypsin with 50 µM riboflavin, 75 µg/ml aminophylline and 0.1 mM thiourea.

as the target molecule *in vitro*. It has already been reported that aminophylline caused a riboflavin-sensitized photohemolysis of human RBC *in vitro*, but had no effect in the absence of riboflavin even after prolonged incubation [36]. Based on the spectral studies of riboflavin and aminophylline we previously suggested that riboflavin upon photoexcitation generates the singlet state, which is partially converted to the triplet state. These singlet and triplet states of riboflavin, by transferring energy to molecular oxygen, give rise to singlet and triplet oxygen species [8,37], which are

very reactive and, in the present case, are likely to attack aminophylline. Aminophylline is probably oxidized to an unknown product, leading to loss of conjugation and hence a decrease in the absorption at 270 nm, the λ_{max} of aminophylline. Hydroxyl radicals are probably generated in the process, since the presence of thiourea, a scavenger of hydroxyl radicals, in the reaction mixture inhibited the oxidation of aminophylline and restored the 270 nm peak to a significant extent (Figure 2). The hydroxyl radical may also be responsible for the damage of trypsin and its fragmentation, as is evident from our results. The proposed mechanism suggesting the formation of hydroxyl radical and its involvement in the inactivation and fragmentation of trypsin is supported by the fact that in all cases, thiourea substantially inhibited the fragmentation and inactivation of trypsin. Other scavenger tested, such as catalase (a scavenger of hydrogen peroxide), SOD (a scavenger of superoxide), and potassium iodide and sodium azide (scavengers of singlet and triplet oxygen, respectively), did not show significant inhibition. The reaction involving riboflavin-mediated photo-damage of aminophylline resulting in the generation of hydroxyl radicals seems to be very different from the previously reported Fenton-like reaction, where a divalent metal ion such as Cu(II) was essentially required for the generation of hydroxyl radicals [9], since in the case of riboflavin-aminophylline, Cu(II) was completely absent from the reaction medium.

With previous reports strongly suggesting aminophylline as an antioxidant, especially against hydroxyl radical [20], and our studies highlighting its hydroxyl radical generation potential when given in combination with riboflavin, care should be taken with patients receiving aminophylline and riboflavin treatments if they are exposed to any form of radiation therapy.

CONCLUSIONS

Based on our results of riboflavin and aminophylline, we strongly suggest that photoilluminated riboflavin generates the singlet and triplet excited states which, in the presence of molecular O_2 via energy transfer, generate $^1O_2^{\cdot}$ and $^3O_2^{\cdot}$ oxygen that attack aminophylline, leading to the oxidation of aminophylline and the generation of hydroxyl radicals in the process. These hydroxyl radicals are possibly involved in the consequent inactivation and fragmentation of trypsin. All this happens in the complete absence of any metal ion such as Cu (II).

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