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LUTEOSKYRIN, AN ANTHRAQUINOID HEPATOTOXIN, AND ASCORBIC ACID GENERATE HYDROXYL RADICAL *IN VITRO* IN THE PRESENCE OF A TRACE AMOUNT OF FERROUS IRON

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Luteoskyrin is a bis-dihydroanthraquinone mycotoxin produced by *Penicillium islandicum* Sopp. By using EPR spin-trap techniques we investigated whether ·OH is formed in a model system containing ascorbic acid and the toxin. In the presence of DMSO and DMPO, we found signals of DMPO-CH_3, a more specific and reliable signature of ·OH than DMPO-OH, together with the signals of ascorbyl radical. DMPO-CH_3 signals increased with time of incubation up to 5.5 min. The DMPO-CH_3 formation depended completely on both lutesokyrin and ascorbic acid, and deferoxamine, an iron-chelator, inhibited its formation. The signals disappeared in the presence of excess amount of catalase whereas SOD showed no effect. These results suggest that ·OH is formed from ferrous ion present in the mixture of H_2O_2 generated from ascrobic acid and luteoskyrin.

KEY WORDS: Hydroxyl radical, DMPO-CH_3, Luteoskyrin, Ascorbic Acid, Ascorbyl radical, Mycotoxin.

INTRODUCTION

Luteoskyrin is a bis-dihydroanthraquinoid mycotoxin produced by *Penicillium islandicum* Sopp[1,2]. It causes acute liver injury characterized by fatty degeneration and centrilobular cell necrosis[1,3]. Long term feeding of the toxin induces hepatoma in experimental animals[4]. The toxin administered through various routes accumulates specifically in liver mitochondria in experimental animals[5,6]. Biochemically, the toxin was found to impairs liver mitochondiral respiration[7] and oxidative phosphorylation[8]. It was also found that the toxin binds to DNA[9] and prevents RNA-synthesis[10] by inhibiting the action of RNA polymerase[11].

We have shown that promethazine, a known free radical scavenger, prevented the induction of the acute liver injury by luteoskyrin[12]. Recently, luteoskyrin was found to transform mammalian cells[13] and enhance ascorbic acid-dependent degradation of deoxyribose and 8-hydroxylation of guanine residues in DNA[14]. Hence it is very likely that certain free radicals are generated from the anthraquinoid mycotoxin in

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the case of development of liver injury. Using a model system which includes various anthraquinones and ascorbic acid, we show in this communication that luteoskyrin, infact, promotes formation of ·OH.

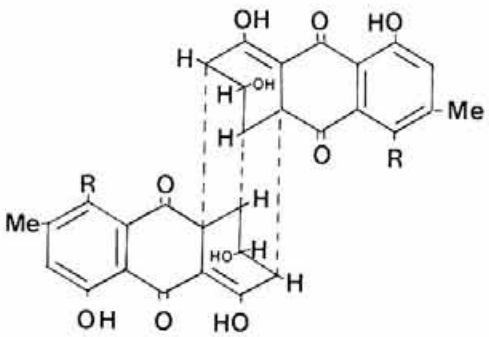
MATERIALS AND METHODS

Materials

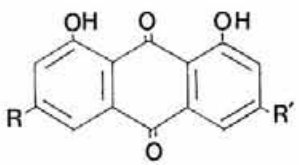
Luteoskyrin, rugulosin and chrysophanol were isolated from cultivated fungal mats of *Penicillium* species. Emodin was isolated from aloe. Danthron was obtained from Wako Chemicals (Kyoto, Japan). Formulas of these anthraquinones were shown in Figure 1. The anthraquinones were purified by column chromatographies. Deferoxamine and DMPO (5,5-dimethyl-1-pyrroline-1-oxide) were obtained from Chiba-Gaigy and Mitsuitoatsu Chem. Co. (Japan), respectively.

EPR Spectrometry

The reaction mixture contained 37 mM potassium phosphate buffer (pH 7.4), 1.9 mM sodium ascorbate, 0.6 M DMSO, 0.08 M DMPO and anthraquinone dissolved in acetone. The final concentration of luteoskyrin and rugulosin were 1.9 mM



LUTEOSKYRIN (R=OH)
RUGULOSIN (R=H)



DANTHRON (R=R'=H)
CHRYSOPHANOL(R=Me,R'=H)
EMODIN(R=Me,R'=OH)

FIGURE 1 Structural formules of luteoskyrin and other anthraquinones.

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and those of emodin, chrysophanol and danthron 3.8 mM, respectively. When anthraquinone was omitted the same volume of acetone was added to the mixture. SOD (1400 unit/ml), catalase (4500 unit/ml), 10 mM deferoxamine or 0.1 mM H_2O_2 was added to the mixture if necessary. The mixture was transfered into a flat quartz EPR cuvette (0.3 mm thickness, 160 μl), which was then fixed in an EPR spectrometer (JEOL, JES FE-2XG). Recording was with 100KHz field modulation (X-band) and started 30 seconds after the addition of anthraquinone to the reaction mixture. Each scan took 2 min.

RESULTS

As shown in Figure 2, 30 seconds after addition of luteoskyrin to the mixture containing ascorbic acid, DMSO and DMPO, small signals of DMPO-CH_3 and the larger signals of ascorbyl radical were observed. Intensities of the former increased with incubation time, while the latter decreased. Without luteoskyrin, only small signals of ascorbyl radical were detected (Figure 2). When ascorbic acid was not included in the mixture, no EPR signal was observed. These results indicate that luteoskyrin promotes formation of DMPO-CH_3 as well as ascorbyl radical. DMPO-CH_3 may be formed from ·OH, DMSO and DMPO. Occurrence of this addduct was often been used to detect ·OH. Catalase but not SOD was found to prevent the formation of DMPO-CH_3 and deferoxamine, a strong iron chelater, also completely inhibited DMPO-CH_3 formation. On the other hand, H_2O_2 added to the reaction mixture increased formation of DMPO-CH_3. Therefore, ·OH seems to be derived from H_2O_2 in the presence of a trace amount of ferrous ion in the mixture. H_2O_2 is known to be formed by the reduction of oxygen coupled with oxidation of ascorbic acid.

Since ·OH reacts rapidly with either DMSO or DMPO to yield DMPO-CH_3 at the expense of DMPO-OH[15], signals for DMPO-OH is expected to appear in the absence of DMPO. However, when DMSO was not present in the reaction mixture no DMPO-OH signal was observed. In a different experiment we found that DMPO-OH signal generated by the modified Fenton reaction disappeared by the addition of ascorbic acid before or after the reaction was started and that only the signals of ascorbyl radical were observed. Therefore, ascorbic acid seems to reduce DMPO-OH to form a compound which can not be detected by EPR spectrometry. The results indicate that detection of ·OH by the formation of DMPO-OH is not possible in the presence of ascorbic acid.

We found previously[14] that the luteoskyrin, rugulosin and danthron but not emodin or chrysophanol enhanced the ascorbic acid -dependent 8-hydroxylation of guanine residues in DNA in the cultured mammalian cells. We tested the possibility that ascorbic acid -dependent ·OH generation is enhanced by the former anthraquinones but not the latter. Upon addition of rugulosin to the mixture containing ascorbic acid, DMSO and DMPO, formation of ascorbyl radical was observed followed by the formation of DMPO-CH_3 adduct. In that case, however, the decrease of the ascorbyl radical and formation of the adduct were much slower than the case of luteoskyrin. Emodin, though to a far lesser degree than luteoskyrin, enhanced the formation of DMPO-CH_3 adduct and danthron and chrysophanol showed almost no effect.

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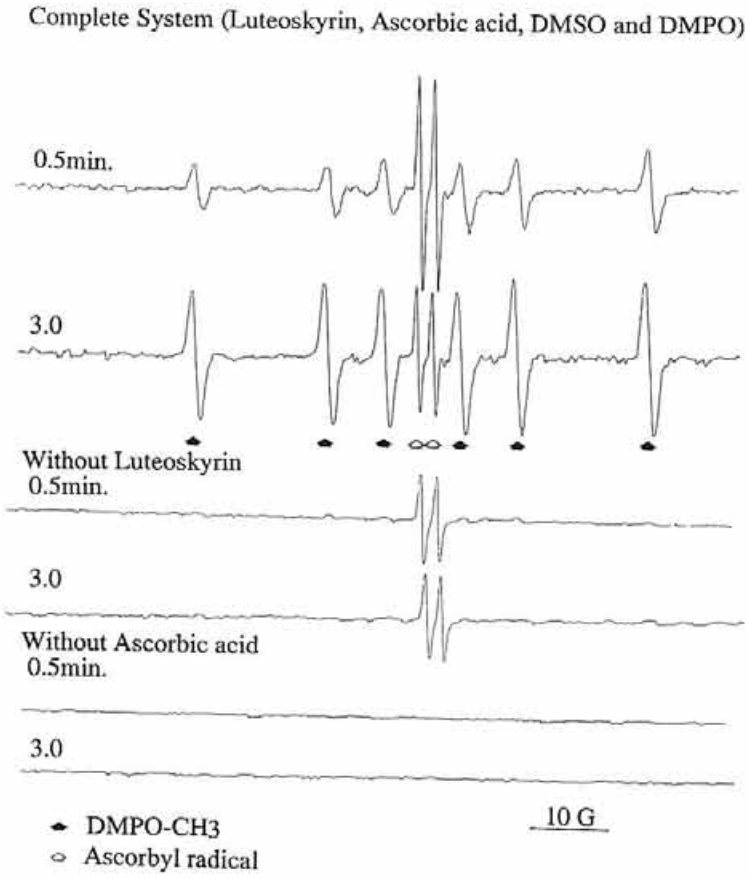

FIGURE 2 EPR spectra of DMPO-CH_3 and ascorbyl radical formed in the mixture containing luteoskyrin, ascorbic acid, DMSO and DMPO.

The complete system contained 37 mM potassium phosphate buffer (pH 7.4), 1.9 mM sodium ascorbate, 0.6 M DMSO, 0.08 M DMPO and 1.9 mM luteoskyrin in acetone. The final concentration of acetone was 1.6 M. In the case without luteoskyrin only acetone was added. The spectra were recorded at 0.5 and 3.0 min after addition of luteoskyrin using the following instrumental conditions: Microwave power 8 mW; microwave frequency, 9.418 GHz; modulation frequency, 100 KHz; modulation ampletute, 0.63 G; magnetic field, 3355 + 50 G; ampletude, 1 × 1000; scan time, 2 min; response, 0.1; and temperature, 25°C. The EPR signals were assigned according to the previously published hyperfine splitting constants: DMPO-CH_3 (A_N = 15.31 G and A_H = 22.00 G)[15]; and ascorbyl radical (A_N = 1.78 G)[17].

DISCUSSION

We have shown previously that luteoskyrin and related anthraquinones stimulate ascorbic acid-dependent 8-hydroxylation of guanine residues in DNA and degradation of deoxyribose[14]. From these evidences and others, we predicted formation of ·OH in the mixture and found that DMPO-CH_3, a strong indicator of ·OH formation, was generated from ascorbic acid, DMSO and DMPO and luteoskyrin. A possible reaction pathway for the formation of DMPO-CH_3 is shown in Figure 3.

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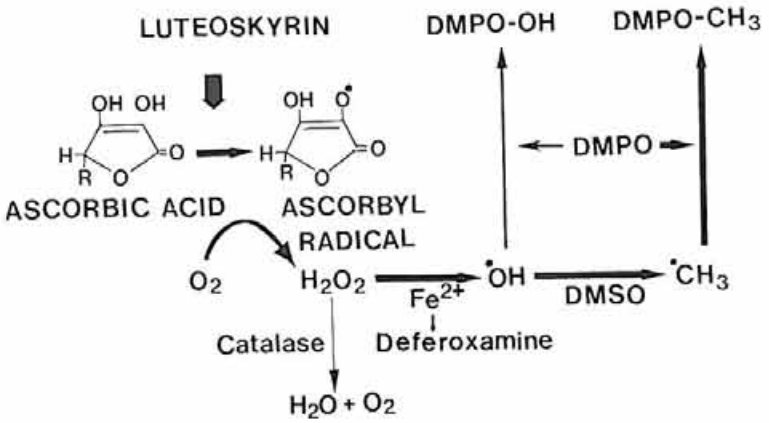

FIGURE 3 A possible mechanism for the formation of DMPO-CH_3 from luteoskysyrin, ascorbic acid, DMSO and DMPO.

Ascorbic acid was essential for the formation of DMPO-CH_3 under the conditions. It is known that ascorbic acid in the aquaous solution is auto-oxidized at neutral pH to form ascorbyl radical[16] accompaning with H_2O_2 formation. Furthermore, catalase and deferoxamine were found to inhibit the formation of DMPO-CH_3 indicating that the ·OH was derived from H_2O_2. ·OH may be formed by a modified Fenton reaction with the aid of a trace amount of ferrous iron which is present in the reaction mixture. Since ascorbyl radical increased immediately after addition of luteoskyrin (Figure 2), luteoskyrin promoted H_2O_2 generation by accelerating the oxidation of ascorbic acid to ascorbyl radical.

Among the anthraquinones examined, luteoskyrin was the most potent accelerator for the DMPO-CH_3 generation followed by rugulosin and emodin. Other monoanthraquinones showed no effect. Further investigations of the correlation between the decrease of the ascorbyl radical and the formation of H_2O_2 are now ongoing. The lower solubilities of the mono-anthraquinones in aqueous solution as compared with luteoskyrin may be one of the reasons of the weak effects of these anthraquinones.

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