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Short Communication

Light-Induced Production of Singlet Oxygen and Superoxide by the Fungal Toxin, Cercosporin[1]

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ABSTRACT

Cercosporin, a toxin produced by members of the fungal genus *Cercospora*, is a photosensitizing compound which rapidly kills plant cells in the light. We have found that cercosporin, when activated by light in the presence of oxygen, is able to generate both singlet oxygen and superoxide ions. Cercosporin, when illuminated in the presence of O_2, reacted with cholesterol to form the 5α-hydroperoxide of cholesterol which is only produced by reaction with singlet oxygen. Cercosporin, in the presence of light, O_2, and a reducing substrate, was also able to reduce *p*-nitro blue tetrazolium chloride, a compound readily reduced by superoxide. Superoxide dismutase, a scavenger of superoxide, inhibited this reaction. Production of both singlet oxygen and superoxide by cercosporin must be considered when studying the possible mechanisms of resistance to cercosporin.

Cercosporin is a toxin produced by members of the genus *Cercospora*, a large group of fungal pathogens which cause damaging leaf spot diseases on a wide range of economically important crops. Cercosporin was first isolated in 1957 by Kuyama and Tamura (11) from *Cercospora kikuchii*, a soybean pathogen. Its characterization and structure were reported independently by Lousberg and co-workers (12) and Yamazaki and Ogawa (20). Cercosporin is toxic to plants, mice, and bacteria (2, 21). It has been shown to cause the peroxidation of plant lipids (3) and to result in major changes in the structure and composition of plant cell membranes (4).

Cercosporin is unique among the known toxins of plant pathogens because it is a photosensitizing compound. Photosensitizers such as cercosporin absorb light to form a long-lived electronically excited state (triplet state) which can then react with molecular oxygen to produce compounds which are toxic to living cells. The triplet sensitizer (^3S) may react with O_2 in several ways (6). It may react with a reducing substrate (R or RH) by the transfer of a hydrogen atom or electron. The resulting sensitizer radical may then react with O_2 to produce superoxide ions $O_2\cdot^-$:[3]

$$R\cdot^+ + S\cdot^- \xleftarrow{R} {}^3S \xleftarrow{RH} SH\cdot + R\cdot$$

$$S\cdot^- \text{ or } SH\cdot \xrightarrow{O_2} O_2\cdot^- \text{ or } HO_2\cdot + S$$

Alternatively, the triplet sensitizer may react directly with O_2 by an energy transfer process yielding the electronically excited singlet state of O_2 ($^1O_2$):

$$^3S \xrightarrow{O_2} {}^1O_2 + S$$

Results obtained from studies on the effects of cercosporin *in vivo* suggested that cercosporin could be damaging plant cells by the production of both $^1O_2$ and $O_2\cdot^-$. First of all, compounds capable of quenching $^1O_2$ are able to prevent the killing of tobacco suspension cultured cells by cercosporin (2). Second, tobacco cell culture mutants (13) selected for resistance to the herbicide paraquat, which generates $O_2\cdot^-$, have decreased sensitivity to cercosporin (unpublished).

Although both $^1O_2$ and $O_2\cdot^-$ are highly reactive and are known to be involved in cellular oxidation reactions, the mechanisms of cellular defense against these compounds are different. Therefore, in order to understand possible resistance mechanisms of plants to cercosporin, it was important to determine which O_2 compounds were being generated. This paper presents evidence that cercosporin, when activated by light in the presence of O_2, is indeed able to generate both $^1O_2$ and $O_2\cdot^-$.

MATERIALS AND METHODS

Cercosporin. Cercosporin was isolated and purified from cultures of *Cercospora nicotianae* as previously described (2).

Photosensitized Oxidation of Cholesterol. Eight mg cercosporin and 500 mg cholesterol (purified by multiple recrystallizations from methanol) were dissolved in 100 ml pyridine and irradiated with a 700-w projector lamp. O_2 was bubbled slowly through the mixture for 5 h. The solvent was removed under reduced pressure, and the residue was dissolved in 50 ml hot methanol. The crystals which separated on cooling were filtered off; the cercosporin remained in the filtrate. The crystals were recrystallized twice from methanol.

The cholesterol 5α-hydroperoxide standard was synthesized by photooxidation with hematoporphyrin by the methods of Ramm and Caspi (14).

UV Oxidation of Cholesterol. Two g of purified cholesterol

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[3] Abbreviations: $O_2\cdot^-$, superoxide ions; $^1O_2$, singlet oxygen; NBT, *p*-nitro blue tetrazolium chloride; SOD, superoxide dismutase.

was dissolved in 20 ml diethyl ether. The mixture was poured into a 15-cm glass Petri plate, and the solvent was allowed to evaporate. The cholesterol was irradiated with a 254 nm UV lamp for 16 h. The irradiated sample was dissolved under N_2 in 40 ml diethyl ether-methanol (1:1) (19). Cholesterol was crystallized from the mixture by cooling, and was filtered off. The remaining solvent containing the oxidation products was reduced to a small volume under reduced pressure.

Thin-Layer Chromatography. Compounds were separated by multiple ascending irrigations on silica gel 60 plates (5 × 20 cm) in three solvent systems, ethyl acetate-heptane (1:1) and hexane-isopropanol (24:1 and 9:1). Standards were dissolved in chloroform (1 mg/ml), and 1- to 5-μl samples were applied to the TLC plates. Oxidation products were made visible after chromatography by spraying the plates with two reagents. *N,N*-Dimethyl-*p*-phenylenediamine (1% w/v) in methanol:H_2O:glacial acetic acid (4.95:4.95:0.1, v/v/v) reacted with the hydroperoxides to form Wurster's red upon air drying or heating (17). After the hydroperoxides were located, the chromatograms were sprayed with 50% H_2SO_4 and heated to develop the characteristic colors of different sterols (18).

IR Spectroscopy. IR spectra were measured on a Perkin-Elmer 621 Grating IR Spectrophotometer with samples prepared in a KBr pellet.

Superoxide Production. Superoxide production was followed using a modification of the photochemical assay described by Giannopolitis and Ries (7) and Beauchamp and Fridovich (1). The reaction mixture consisted of 3.9 μM cercosporin or 1.3 μM riboflavin, 13 mM methionine, 63 μM NBT, 50 mM sodium carbonate (pH 10.2) in a final volume of 3 ml. SOD (Sigma) was added at 1 mg (2800 units) per ml of the reaction mixture. The mixture was irradiated by passing the beam of a 700-w tungsten projector lamp through a $CuSO_4$ solution. Superoxide production (reduction of NBT) was monitored by following the increase in *A* at 560 nm.

RESULTS AND DISCUSSION

Analysis of oxidation products of cholesterol is one of the best ways of distinguishing $^1O_2$ production from the production of radical oxidizers such as $O_2\cdot^-$. The reaction of $^1O_2$ with cholesterol results primarily in the production of the 5α-hydroperoxide of cholesterol (10). Radical oxidations, such as those by $O_2\cdot^-$, produce many different products (primarily the 7α- and 7β-hydroperoxides), but have never been shown to produce the 5α-hydroperoxide (19). Therefore, positive identification of the 5α-hydroperoxide after reaction of cercosporin with cholesterol in the light provides strong evidence that $^1O_2$ is generated by cercosporin.

Photooxidation of cholesterol by cercosporin yielded one product which co-chromatographed in three different solvent systems with the 5α-hydroperoxide standard. The product was identified as a hydroperoxide by its formation of Wurster's red with *N,N*-dimethyl-*p*-phenylenediamine (17). The purified product had a melting point of 145 to 147°C which agrees with published values for the 5α-hydroperoxide (16). IR spectroscopy showed the presence of absorption bands at 1640, 1045, 1025, 828, 810, 797, 760, and 743 cm^{-1} which are characteristic of the 5α-hydroperoxide (15). Furthermore, the addition of β-carotene, a $^1O_2$ quencher, to the reaction mixture completely inhibited the production of the 5α-hydroperoxide by cercosporin. By contrast, UV oxidation of cholesterol formed several different products, none of which could be identified as the 5α-hydroperoxide by co-chromatography with the 5α-hydroperoxide standard or by the formation of Wurster's red.

These results demonstrate that cercosporin-sensitized photooxidations can occur via the production of $^1O_2$. The production of $^1O_2$ by cercosporin has recently been confirmed in another laboratory by luminescence at 1270 nm following pulsed-laser excitation (5). Thus, it is very likely that $^1O_2$ is largely responsible for cercosporin toxicity to plant tissues. This is supported by the already mentioned observation that $^1O_2$ quenchers can inhibit the killing of cultured tobacco cells by cercosporin (2).

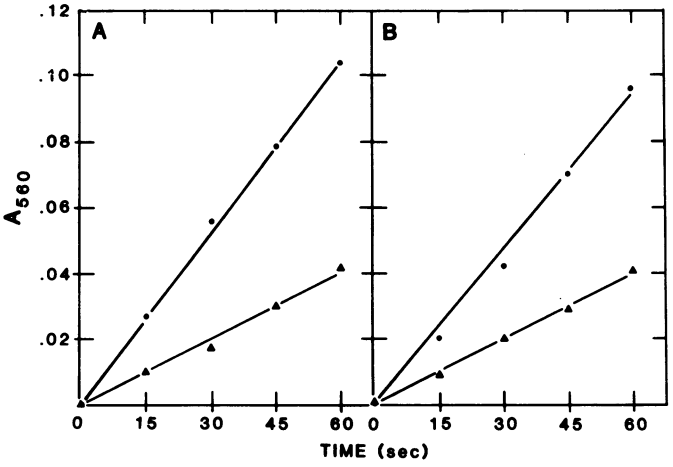

FIG. 1. Production of $O_2\cdot^-$ by (A) riboflavin and (B) cercosporin with (▲) and without (●) the addition of SOD. The reaction mixture contained 1.3 μM riboflavin or 3.9 μM cercosporin, 13 mM methionine, 63 μM NBT, 50 mM sodium carbonate (pH 10.2), and 1 mg (2800 units) per ml SOD. $O_2\cdot^-$ production (NBT reduction) was monitored by following the increase in *A* at 560 nm.

Production of $O_2\cdot^-$ by cercosporin was demonstrated through the use of an assay developed to measure SOD. The standard assay utilizes riboflavin as a photosensitizer and methionine as a reducing substrate. The substitution of cercosporin for riboflavin as the photosensitizer resulted in the production of superoxide (reduction of NBT) (Fig. 1), although 3 times as much cercosporin as riboflavin was required to give comparable results. The addition of the superoxide-scavenging enzyme SOD to the reaction mixture inhibited both the riboflavin- and cercosporin-induced NBT reduction to comparable levels. By contrast, $^1O_2$ quenchers such as β-carotene and 1,4-diazabicyclo octane had no effect on the cercosporin-induced reduction of NBT (data not shown). It is important to note that the production of $O_2\cdot^-$ by cercosporin required the presence of the reducing substrate methionine (see "Introduction" for mechanism). Since reducing substrates are readily available in living tissues, it is possible that cercosporin is damaging cells by $O_2\cdot^-$ as well as by $^1O_2$.

The production of both $^1O_2$ and $O_2\cdot^-$ has important consequences for the understanding of possible resistance mechanisms to cercosporin. Resistance to $O_2\cdot^-$-generating agents is known. For example, cultivars of rye grass resistant to paraquat have significantly higher levels of SOD, catalase, and peroxidase than paraquat-sensitive lines (8). Resistance to $^1O_2$, however, may be much more difficult. $^1O_2$ is quenched very efficiently by carotenoids (9), and carotenoids in plants are effective quenchers of photooxidation processes sensitized by Chl (6). However, plants are not usually resistant to the action of exogenous sensitizers and, in fact, all plants which have been tested are sensitive to cercosporin. Furthermore, we have been unable to select for cercosporin-resistant cells by the mutagenesis and selection of haploid tobacco protoplasts in culture (unpublished). This may be due to the fact that carotenoids in plants are primarily localized in plastids, and in this location they might be unable to protect against $^1O_2$ produced in other parts of the cell.

Although plants, mice, and bacteria are very sensitive to cercosporin, *Cercospora* species themselves produce large quantities of the toxin in the light and are apparently unaffected by it. This

resistance appears to be a fairly general phenomenon in fungi, for our preliminary studies suggest that a number of different fungi are also resistant (unpublished). A study of fungal resistance mechanisms may provide information which will help in the future selection or engineering of cercosporin-resistant plants.

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