APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1989, p. 7–14
0099-2240/89/010007-04$02.00/0

Vol. 55, No. 1

Photodynamic Production of Superoxide In Vitro by Altertoxins in the Presence of Reducing Agents†

PHILIP E. HARTMAN,[1]* CAROLYN K. SUZUKI,[1] AND MICHAEL E. STACK[2]

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218,[1] and Division of Contaminants Chemistry, Food and Drug Administration, Washington, DC 20204[2]

Received 11 July 1988/Accepted 30 September 1988

Superoxide production by the three 4,9-dihydroxyperylene-3,10-quinone fungal toxins, altertoxins I, II, and III, was stimulated on illumination with broad-spectrum light. As determined previously for cercosporin, superoxide production by illuminated altertoxins was increased by the addition of the reducing substances ergothioneine or urate; ascorbate also effectively increased superoxide production. Illuminated urate alone engendered some superoxide production.

Plant pathogenic fungi of the genus *Alternaria* produce a variety of toxins, among which are the altertoxins I, II, and III (14, 21, 25). The three altertoxins (Fig. 1) are 4,9-dihydroxyperylene-3,10-quinones (25). The altertoxins are structurally similar to cercosporin and several other fungal toxins (19, 27, 28). Cercosporin is a primary toxin of the plant pathogenic fungal genus *Cercospora*, the cause of leaf spot disease (for a review, see reference 6). Cercosporin is photodynamic and produces singlet oxygen when illuminated (5, 6, 8); this behavior may account in part for dependence on light intensity for the primary toxicity of cercosporin for tissue culture cells in vitro (5) and for the light intensity dependence of the toxicity of the fungus for plants in vivo (3). Daub and Hangarter (7) showed that cercosporin also produces superoxide anion when it is illuminated in the presence of the reducing agent methionine. An alternate reducing agent, ergothioneine, significantly quenched singlet oxygen production and enhanced superoxide production by illuminated cercosporin (10). Both singlet oxygen and superoxide generation are positively light regulated.

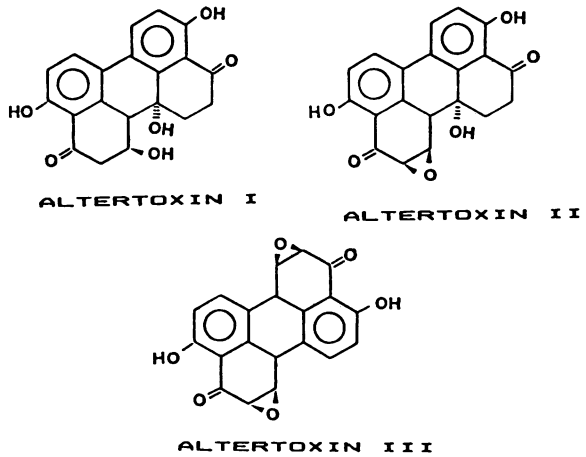

FIG. 1. Structures of altertoxins I, II, and III (25, 26).

Here we demonstrate the production of superoxide by altertoxins I, II, and III, a process that is strongly photo facilitated and that is stimulated by reducing agents.

MATERIALS AND METHODS

Reagents. Sodium carbonate, ascorbate, ergothioneine, urate, Nitro Blue Tetrazolium (NBT), and superoxide dismutase (SOD; EC 1.15.1.1; lyophilized powder derived from bovine blood, 3,000 U, 0.9 mg) were obtained from Sigma Chemical Co., St. Louis, Mo. Copper sulfate and dimethyl sulfoxide were from J. T. Baker Chemical Co., Phillipsburg, N.J. Altertoxins, I, II, and III were extracted and purified from cultures of *Alternaria alternata*, as described by Stack and Prival (26). Cercosporin was isolated and purified from cultures of *Cercospora nicotianae* as described previously (5) and was kindly provided by Margaret E. Daub (North Carolina State University at Raleigh). Na_2CO_3 buffer (0.05 M, pH 10.2) was used to make stock solutions of the reducing agents, NBT and SOD, at 1 mM, 1.6 mM, and 1 mg/ml. Ascorbate was freshly prepared before each experiment, in order to minimize autooxidation. Altertoxins were prepared at a concentration of 1 mM in 33% dimethyl sulfoxide in Na_2CO_3 buffer. All stock reagents were refrigerated at 4°C and brought to room temperature before each experiment.

NBT reduction. NBT reduction was used as an indicator of superoxide production (1). Experimental samples were mixed directly in disposable polystyrene cuvettes (PGC Scientifics, Gaithersburg, Md.), such that the reaction mixture contained 80 μM NBT and 10 μM altertoxin I, II, or III, in addition to 10 μM ascorbate and 10 or 100 μM ergothioneine or urate, when assayed. Additionally, 10 μg of SOD per ml was present when necessary to inhibit superoxide reduction of NBT. Controls contained the same reagents as experimental samples, but without NBT; each sample was brought to a total volume of 4 ml with Na_2CO_3 buffer (0.05

TABLE 1. Rates of absorption changes (560 nm) of altertoxins with and without illumination

Illumination	Rate of absorption change of:		
	Altertoxin I	Altertoxin II	Altertoxin III
Dark	−1.0 ± 0.2 (2)[a]	−1.9 ± 0.1 (2)	−0.8 ± 0.4 (2)
Light	−1.0 ± 0.1 (2)	−3.7 ± 0.4 (3)	−1.5 ± 0.1 (3)

[a] Values in parentheses are the number of repeated assays.

* Corresponding author.

† Contribution 1419 of the Department of Biology, The Johns Hopkins University, Baltimore, MD 21218.

TABLE 2. Rates of NBT reduction (560 nm) by reducing agents in the absence and in the presence of added SOD

Reducing agent	Rate of NBT reduction under:	
	Light	Dark
Ascorbate		
10 μM	0.9 ± 0.4 (2)[a]	
10 μM + SOD	−0.7 ± 0.1 (2)	
Ergothioneine		
10 μM	0.3 ± 0.3 (2)	
10 μM + SOD	0.0 ± 0.0 (2)	
100 μM	0.5 ± 0.1 (3)	
100 μM + SOD	−0.2 ± 0.2 (2)	
Urate		
10 μM	2.0 ± 0.0 (3)	0.8 ± 0.1 (2)
10 μM + SOD	0.0 ± 0.0 (2)	0.0 ± 0.0 (2)
100 μM	4.3 ± 0.5 (4)	1.1 ± 0.2 (2)
100 μM + SOD	0.1 ± 0.1 (3)	0.1 ± 0.1 (2)

[a] Values in parentheses are the number of repeated assays.

M, pH 10.2). Reactions were carried out at room temperature (25°C), and the oxygen present in solution was that dissolved from air without bubbling. As indicated, samples were kept in the dark or illuminated with a 150-W projector spotlamp (tungsten filament; Sylvania), which was suspended 26 cm above the surface on which the samples were placed. The light passed through a 2% $CuSO_4$ solution, in order to absorb the heat generated by the lamp. The light intensity was 1.1 millieinsteins per m^2 per s, as measured at the midpoint of the cuvette with a quantum photometer (LI-185; Li-Cor). Optical densities (ODs) were followed at 560 nm in a dual-beam spectrophotometer (series 634; Varian Instruments, Inc.). All rates are expressed as (dy/dt ± standard error) × 10^{-4}.

RESULTS

There was a slow loss of the A_{560} of the altertoxins in carbonate buffer at pH 10.2 in the dark; this loss in OD was enhanced by illumination in the cases of altertoxins II and III (Table 1). The instability of altertoxins in solutions containing dimethyl sulfoxide has been noted previously (26). Therefore, experimental samples were compared with parallel samples containing altertoxin alone. When this procedure is used, it is assumed that the rate of spontaneous absorbance loss and photobleaching of altertoxins is analogous in cuvettes containing additional reactants, an assumption that may or may not be true. For example, additional reactants could alleviate photobleaching of altertoxins, a possibility that was taken into account in the interpretation of results.

Table 2 presents data describing interactions of reducing agents (ascorbate, ergothioneine, and urate) with NBT in the absence of altertoxins. Like ascorbate, ergothioneine (references in reference 11) and urate (references in reference 13) are effective reducing agents. In all cases there was some reduction of NBT, as measured by an increase in the OD at 560 nm, and this reduction of NBT by illuminated reducing agents was strongly suppressed by the addition of SOD. Ascorbate was tested only at 10 μM, because concentrations above this level led to the almost instantaneous reduction of NBT. Reduced glutathione also instantaneously reduced NBT at all concentrations tested. Of particular interest (Table 2) was the concentration-dependent production of superoxide by illuminated urate, indicating that urate is, itself, a photo-facilitated producer of superoxide. The rates of reduction by illuminated reducing agents alone have not been subtracted from data from experiments in which altertoxins were examined (Table 3).

Data in Table 3 indicate that illuminated altertoxins produced superoxide when they were illuminated in the absence of added reducing agents. This superoxide production was enhanced in the presence of reducing agents (Table 3). Figure 2 shows results of representative individual experiments in which the reduction of NBT was followed during illumination of altertoxins I, II, and III, in reaction mixtures containing 100 μM ergothioneine in the absence and the presence of 10 μg of SOD per ml. Figure 3 shows results of an individual experiment in which altertoxin II was illuminated in the absence of added reducing agent and in the presence of 10 μM ascorbate, urate, and ergothioneine. The rate of NBT reduction by altertoxin and reducing agent with illumination immediately fell to zero when samples were transferred to the dark; on reintroduction to the light, the original rate of reduction was established once again (data not shown). Data in Tables 1 to 3 were compiled from replicate experiments analogous to those shown in Fig. 2 and 3. As in Fig. 2 and 3, rates of NBT reduction did not deviate from linearity with time over the 60-min assay period in each of the individual cases tabulated in Table 3.

Cercosporin, which has been tested previously for superoxide generation by a procedure that was slightly different

TABLE 3. Rates of NBT reduction (560 nm) by altertoxins with illumination

Treatment	Rate of NBT reduction by:		
	Altertoxin I	Altertoxin II	Altertoxin III
Altertoxin alone	4.9 ± 0.4 (11)[a]	5.4 ± 0.4 (9)	2.2 ± 0.5 (12)
SOD (10 μg/ml)	0.5 ± 0.1 (4)	1.4 ± 0.3 (5)	0.0 ± 0.2 (2)
Ascorbate (10 μM)	7.5 ± 0.4 (6)	8.5 ± 1.2 (6)	4.5 ± 1.4 (4)
Ascorbate (10 μM) + SOD (10 μg/ml)	0.9 ± 0.5 (3)	1.7 ± 0.6 (3)	0.6 ± 0.6 (2)
Ergothioneine (10 μM)	12.0 ± 2.0 (2)	13.7 ± 1.8 (3)	7.2 ± 0.9 (2)
Ergothioneine (10 μM) + SOD (10 μg/ml)	0.8 ± 0.1 (2)	1.3 ± 0.2 (2)	0.2 ± 0.3 (3)
Ergothioneine (100 μM)	29.8 ± 3.8 (4)	34.0 ± 1.0 (2)	24.5 ± 11.5 (2)
Ergothioneine (100 μM) + SOD (10 μg/ml)	8.0 ± 1.4 (2)	5.3 ± 0.1 (2)	2.8 ± 0.1 (2)
Urate (10 μM)	7.8 ± 1.2 (2)	10.3 ± 0.4 (3)	6.1 ± 0.8 (2)
Urate (10 μM) + SOD (10 μg/ml)	1.3 ± 0.3 (2)	1.2 ± 0.1 (2)	1.2 ± 0.8 (2)
Urate (100 μM)	22.5 ± 4.5 (2)	23.0 ± 5.0 (2)	8.2 ± 2.8 (2)
Urate (100 μM) + SOD (10 μg/ml)	5.4 ± 0.6 (2)	5.5 ± 0.9 (2)	2.8 ± 0.6 (2)

[a] Values in parentheses are the number of repeated assays.

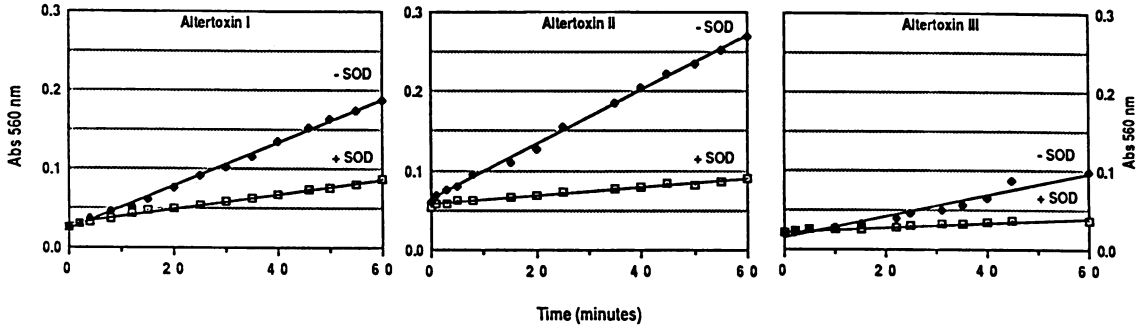

FIG. 2. Reduction of NBT by illuminated altertoxins in the presence of 100 μM ergothioneine, with and without 10 μg of SOD per ml.

from that used here (10), was reexamined under conditions identical to those used for the altertoxins. In duplicate experiments, illuminated 10 μM cercosporin alone engendered an average rate [$(dy/dt) \times 10^{-4}$] of NBT reduction of 20.5, and this was essentially eliminated by the addition of 10 μg of SOD per ml. In the presence of 100 μM ergothioneine, illuminated 10 μM cercosporin reduced NBT at an average rate of 155.5 in the absence of SOD and 33.0 in the presence of SOD. Thus, cercosporin appeared to be more effective than any of the altertoxins in releasing photo-mediated superoxide.

DISCUSSION

Our data provide evidence that illuminated altertoxins I, II, and III behave analogously to illuminated cercosporin (7, 10). Namely, there is a photo-facilitated production of superoxide that is strongly enhanced in the presence of reducing agents. This enhancement by electron donors is marginal in the presence of 10 μM ascorbate, 10 μM urate, and, in the case of altertoxin III, the presence of 100 μM urate (Table 3). By "marginal' we mean that the changes in OD observed could, in these cases, be due to a combination of superoxide production elicited by the illuminated reducing agent itself and simultaneous quenching by the reducing agent of altertoxin photobleaching. However, in the cases of 10 and 100 μM ergothioneine and 100 μM urate (the latter for altertoxins I and II), there was a strikingly high rate of NBT reduction that was essentially eliminated by the addition of SOD (Table 3). For example, by subtracting the effect of reducing agent alone (Table 2) and by assuming that the reducing agent completely prevents altertoxin photobleaching (Table 1), there were still rates of 28.3, 29.8, and 22.5 for altertoxins I, II, and III, respectively, in the presence of 100 μM ergothioneine. This high rate of NBT reduction was light dependent. We conclude that the three altertoxins can generate superoxide and that this generation is strongly enhanced by illumination in the presence of an appropriate reducing agent.

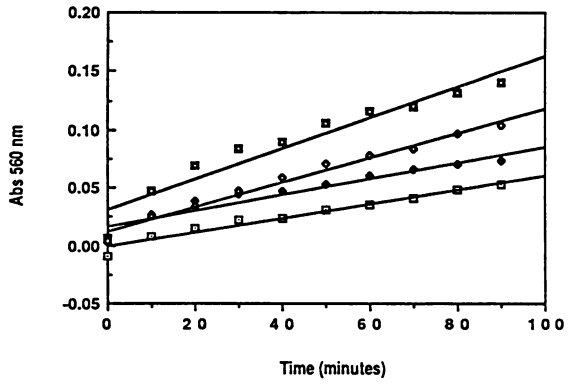

FIG. 3. Reduction of NBT by illuminated altertoxin II (10 μM) in the absence of added reducing agents (⊡) and in the presence of 10 μM ergothioneine (◘), urate (◇), and ascorbate (◆).

It seems logical to assume that the production of superoxide anion is at least predominantly generated by the reduction of the fungal quinones to semiquinones (or to hydroquinones) which then, in a photo-facilitated process, react with dioxygen to produce superoxide anion (redox cycling; for a review, see reference 24). A second possibility is the generation of singlet oxygen, (see below), with the subsequent one-electron transfer to the singlet oxygen, forming superoxide (cf. reference 20). Finally, a more involved sequence of reactions may occur involving radicals formed from the reducing agents themselves (the reactions are summarized in reference 18).

Some superoxide-generating quinones (e.g., 1,4- and 1,2-naphthoquinones) inhibit SOD activity (23), but we found no evidence that such an inactivation occurs during superoxide formation mediated by the altertoxins. SOD remained active throughout the period of our experiments (Fig. 2). Redox cycling by the dihydroxyperylene quinones would be expected to occur in the dark as well under the appropriate reducing conditions; this may account for the residual dark toxicity of cercosporin noted by Macri and Vianello (17), although activation to a toxic arene oxide is also possible (25).

Besides producing superoxide in the presence of reducing agents (7, 10), illuminated cercosporin generates singlet oxygen with a high quantum yield in the absence of reducing agents (5, 8). Singlet oxygen production may be generated by the formation and spontaneous decay of an anthracene endoperoxide within the perylenequinone structure; photomediated formation of such an endoperoxide has been documented for the related fungal pigment hypocrellin (4). While so far untested, it would be surprising if the altertoxins did not also form endoperoxides and release singlet oxygen photodynamically. We propose that the generation of singlet oxygen and superoxide in fluctuating amounts may overcome plant host defenses against oxidative damage, defenses that are individually focused on particular oxidative species (e.g., see references 9 and 22).

It also seems likely that plants use chemical arsenals in their defense that mimic those applied by pathogenic fungi.

Many plants contain α-hydroxy quinones at high concentrations in particular tissues (27, 28). For example, the plant and fungal naphthoquinone juglone produces superoxide in susceptible cells (12). One extended quinone of plant origin, hypericin, is photodynamic, produces singlet oxygen when illuminated, and, in the presence of methionine, generates superoxide (15). Photodynamic α-hydroxy quinones may constitute one predominant class of pesticides produced by nature.

Finally, the fungal production of photodynamic toxic secondary products is, to a great extent, environmentally controlled. It is striking that cercosporin production by at least some strains of *Cercospora* cultured in vitro is stimulated by light, oxygen, and ascorbate (2, 16). This suggests that generation of superoxide may feed back in some manner and lead to increased toxin production in an optimal environment containing appropriate reducing agents (10).

LITERATURE CITED

1. **Auclair, C., and E. Voisin.** 1985. Nitroblue tetrazolium reduction, p. 123–132. *In* R. A. Greenwald (ed.), CRC handbook for oxygen radical research. CRC Press, Boca Raton, Fla.
2. **Balis, C., and M. G. Payne.** 1971. Triglycerides and cercosporin from *Cercospora beticola*: fungal growth and cercosporin production. Phytopathology **61**:1477–1484.
3. **Calpouzos, L., and G. F. Stallknecht.** 1967. Symptoms of cercospora leaf spot of sugar beets influenced by light intensity. Phytopathology **57**:799–800.
4. **Chen, W.-s., Y.-t. Chen, X.-y. Wan, E. Friedrichs, H. Puff, and E. Breitmaier.** 1981. Die Struktur des Hypocrellins und seines Photooxidationsproduktes Peroxyhypocrellin. Liebigs Ann. Chem. **1981**:1880–1885.
5. **Daub, M. E.** 1982. Cercosporin, a photosensitizing toxin from *Cercospora* species. Phytopathology **72**:370–374.
6. **Daub, M. E.** 1987. The fungal photosensitizer cercosporin and its role in plant disease, p. 271–280. *In* J. R. Heitz and K. R. Downum (ed.), Light-activated pesticides. American Chemical Society, Washington, D.C.
7. **Daub, M. E., and R. P. Hangarter.** 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. Plant Physiol. **73**:855–857.
8. **Dobrowolski, D. C., and C. S. Foote.** 1983. Cercosporin, a singlet oxygen generator. Angew. Chem. Int. Ed. Engl. **22**:720–721.
9. **Elstner, E. F.** 1982. Oxygen activation and oxygen toxicity. Annu. Rev. Plant Physiol. **33**:73–96.
10. **Hartman, P. E., W. J. Dixon, T. A. Dahl, and M. E. Daub.** 1988. Multiple modes of photodynamic action by cercosporin. Photochem. Photobiol. **47**:699–703.
11. **Hartman, Z., and P. E. Hartman.** 1987. Interception of some direct-acting mutagens by ergothioneine. Environ. Mol. Mutagen. **10**:3–15.
12. **Hassan, H. M., and I. Fridovich.** 1979. Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. Arch. Biochem. Biophys. **196**:385–395.
13. **Hochstein, P., L. Hatch, and A. Sevanian.** 1984. Uric acid: functions and determination. Methods Enzymol. **105**:162–166.
14. **King, A. D., Jr., and J. E. Schade.** 1984. *Alternaria* toxins and their importance in food. J. Food Prot. **47**:886–901.
15. **Knox, J. P., R. I. Samuels, and A. D. Dodge.** 1987. Photodynamic action of hypericin, p. 265–270. *In* J. R. Heitz and K. R. Downum (ed.), Light-activated pesticides. American Chemical Society, Washington, D.C.
16. **Lynch, F. J., and M. J. Geoghegan.** 1979. Regulation of growth and cercosporin photoinduction in *Cercospora beticola*. Trans. Br. Mycol. Soc. **73**:311–327.
17. **Macri, F., and A. Vianello.** 1981. Inhibition of K^+ uptake and H^+ extrusion caused by non-irradiated cercosporin. Plant Sci. Lett. **22**:29–37.
18. **Martin, J. P., and N. Logsdon.** 1987. Oxygen radicals are generated by dye-mediated intracellular photooxidations: a role for superoxide in photodynamic effects. Arch. Biochem. Biophys. **256**:39–49.
19. **Merlini, L., and G. Nasini.** 1983. Natural perylenequinones from moulds, p. 121–137. *In* C. Szantay, A. Gottseger, and G. Kovacs (ed.), Chemistry and biotechnology of biologically active natural products. Elsevier, New York.
20. **Saito, I., T. Matsuura, and K. Inoue.** 1983. Formation of superoxide ion via one-electron transfer from electron donors to singlet oxygen. J. Am. Chem. Soc. **105**:3200–3206.
21. **Schade, J. E., and A. D. King, Jr.** 1984. Analysis of the major *Alternaria* toxins. J. Food Prot. **47**:978–995.
22. **Schmidt, A., and K. J. Kunert.** 1987. Antioxidative systems: defense against oxidative damage in plants. UCLA Symp. Mol. Cell. Biol., New Ser. **48**:401–413.
23. **Smith, M. T., and C. G. Evans.** 1984. Inhibitory effect of superoxide-generating quinones on superoxide dismutase. Biochem. Pharmacol. **33**:3109–3110.
24. **Smith, M. T., C. G. Evans, H. Thor, and S. Orrenius.** 1985. Quinone-induced oxidative injury to cells and tissues, p. 91–113. *In* H. Sies (ed.), Oxidative stress. Academic Press, Inc., New York.
25. **Stack, M. E., E. P. Mazzola, S. W. Page, A. E. Pohland, R. J. Highet, M. S. Tempesta, and D. G. Corley.** 1986. Mutagenic perylenequinone metabolites of *Alternaria alternata*: altertoxins, I, II, and III. J. Natl. Proc. **49**:866–871.
26. **Stack, M. E., and M. J. Prival.** 1986. Mutagenicity of the *Alternaria* metabolites altertoxins I, II, and III. Appl. Environ. Microbiol. **52**:718–722.
27. **Thomson, R. H.** 1971. Naturally occurring quinones, 2nd ed. Academic Press, Inc., New York.
28. **Thomson, R. H.** 1987. Naturally occurring quinones. III. Recent advances. Chapman Hall, New York.