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Cytotoxic Effects of Singlet Oxygen

by Leonard J. Schiff,* Walter C. Eisenberg,† John Dziuba,† Kevin Taylor,† and Steven J. Moore*

The toxic effects of gas-phase singlet oxygen, $^1O_2$, on the ciliated respiratory epithelium of hamster trachea have been demonstrated. Tracheal explants treated with $^1O_2$ showed a dose-dependent decrease in cilia beating frequency and focal ciliostasis. A statistically significant decrease in ciliary activity occurred at $^1O_2$ concentrations as low as 154 ppb after a 2-hr exposure. Cytological alterations in the mucociliary epithelium were observed in explants exposed to 235 ppb $^1O_2$ or greater. When cytotoxic effects were related to the time of exposure to $^1O_2$, maximum effects occurred after a 4-hr exposure. *In vitro* recovery studies indicate that ciliary activity returned to normal between 4 and 8 hr after exposure.

Introduction

An on-going objective of the work in this laboratory is to investigate the role of oxidants in environmental health. Toward this end, a number of studies have been initiated to more fully define the chemical and biological effects of individual oxidant species, especially of singlet molecular oxygen ($^1O_2$). Evidence for the toxicity of singlet oxygen has been based primarily on the observed reactivity of this oxidant with biological substrates and on experiments using sensitizers in which $^1O_2$ may not be the only toxic species generated (*1–3*). A generator for forming $^1O_2$ in the gas phase at atmospheric pressure was developed in our laboratory several years ago. This generator provides a clean source of $^1O_2$ for laboratory experiments. We have shown that gas-phase $^1O_2$ is cytotoxic (*4*) and induces unscheduled DNA synthesis (*5*) and sister chromatid exchange (unpublished results). These observations take on particularly important significance in light of the recent report (*6*) suggesting that prior use of photoradiation therapy may lead to cell damage and consequently resistance to the use of this promising cancer treatment method. It is now widely accepted that photoradiation therapy involves the production of $^1O_2$ which is responsible for the cytocidal action (*7*).

In the dose-range study just completed, we show the effect of $^1O_2$ on tracheal organ cultures of hamster respiratory epithelium over a relatively wide range of $^1O_2$ concentrations. The time required for the respiratory epithelium to recover from exposure to $^1O_2$ was also studied.

*Life Sciences Department, IIT Research Institute, 10 West 35th Street, Chicago, IL 60616.
†Chemistry and Chemical Engineering Department, IIT Research Institute, 10 West 35th Street, Chicago, IL 60616.

Methods

Preparation of Tracheal Organ Culture

Tracheal organ cultures were prepared from 4- to 6-week-old male Syrian golden hamsters, strain HSD:(SYR)BR (Harlan Sprague-Dawley, Indianapolis, IN), and maintained in culture as previously described (*8*). Tracheal rings were initially incubated in groups of eight per 60-mm dish. The culture medium consisted of 25 mM Hepes buffered CMRL 1066 medium (GIBCO, Grand Island, NY) supplemented with 2% fetal bovine serum (Sterile Systems, Inc., Logan, UT), 2 mM L-glutamine (GIBCO), and 50 μg Garamycin/mL (M.A. Bioproducts, Walkersville, MD).

Generation of Singlet Oxygen and Exposure System

Singlet oxygen was generated by the method of heterogeneous photosensitization (*9,10*). Details of the $^1O_2$ generation and exposure apparatus are described elsewhere (*5*). The $^1O_2$ generator consisted of a 13-mm OD × 45-cm water-jacketed Pyrex flow tube lined with a thin film of Rose Bengal that was prepared by evaporation from a methanol solution of the dye. A mixture of 98% N_2/2% O_2 was passed through the flow tube at 8.2 L/min while the dye film was exposed to strong visible radiation from four 1000-watt projection lamps (G.E. Model DPT) enclosed in an air-cooled reflector. The exit gas was passed directly into the top of a cube-shaped exposure chamber (1 × 1 × 1 ft) and down onto culture dishes supported on a rack in the middle of the chamber.

The concentration of the gas-phase $^1O_2$ entering the chamber was measured using a liquid nitrogen-cooled germanium photodiode that monitors the 1.27-μm emis-

Table 1. Effects of 2-hr exposure to $^1O_2$ on tracheal epithelium *in vitro*.[a]

Treatment, $^1O_2$, ppb	Mean cilia beating frequency, beats/min				Mean % normal epithelium			
	Background	SE	Postexposure	SE	Background	SE	Postexposure	SE
Control	1223	3	1197	4	97	1	90	2
414	1215	5	793	90	91	2	58*	7
Control	1242	4	1202	5	97	2	89	2
403	1234	3	911*	26	93	2	56*	3
Control	1251	5	1199	5	96	3	92	1
345	1240	4	994*	16	93	1	62*	5
Control	1236	4	1188	6	98	1	99	2
290	1246	4	1026*	16	94	1	64*	7
Control	1225	5	1195	3	94	2	89	2
266	1231	5	1057*	10	93	1	64*	3
Control	1263	6	1204	4	96	1	90	2
235	1233	4	1119*	11	95	2	63*	3
Control	1233	5	1194	6	95	1	91	2
154	1227	4	1140*	9	93	1	80	4
Control	1246	4	1198	7	95	3	89	3
97	1228	5	1181	5	95	2	86	2

[a] Singlet oxygen treatment and control values represent means ± SE from eight separate ring cultures.
* Significantly different from 0-time baselines values and the blank postexposure ($p < 0.05$; χ^2 distribution test for percent normal epithelium, $p < 0.05$; Dunnett's test for beating frequency).

sion of $^1O_2$ (*9,10*). The optical detector was calibrated in trapping experiments using the $^1O_2$-tetramethylene reaction. The limit of detection of $^1O_2$ in the gas phase using this reaction is 2.5 ppb. Control exposures were performed in which the generator lights were not turned on. These experiments were identical to the $^1O_2$ exposures except no $^1O_2$ was present in the 98% N_2/2% O_2 gas, as evidenced by failure to observe the 1.27 μm emission of $^1O_2$ during control experiments.*

Three uncovered culture dishes, each containing eight ring explants with 2 mL of L-15 medium (GIBCO), were placed in an atmospheric chamber (Bellco Glass, Inc., Vineland, NJ). The chamber was placed on a rocker platform (Bellco Glass, Inc.) that rocked at 10 cycles/min, allowing the explants to contact both $^1O_2$ and L-15 medium. Evaporation loss was corrected by the addition of medium to the dishes after each hour of exposure.

Experimental Design

In the first series of experiments, we determined the effect of increasing $^1O_2$ concentration on the ciliary activity and epithelial cytology of hamster tracheal cultures by examining each of the eight ring explants after a culture period of 48 hr. Sets of tracheal cultures were then exposed for a 2-hr period to a mixture of nitrogen and oxygen gas containing $^1O_2$ concentrations of 97, 154, 235, 266, 290, 345, 403, or 414 ppb. Immediately after exposure, we determined the alterations in the cilia beating frequency and cytology by light microscopy. For each singlet oxygen exposure concentration, a control experiment was performed in which eight ring explants were exposed for 2 hr to the nitrogen/oxygen gas stream without $^1O_2$.

In the second series of experiments, we determined the time required for hamster tracheal cultures to recover from a 2-hr exposure to 401 ppb $^1O_2$. Eight explants were examined at 1, 2, 4, and 8 hr after exposure for 2 hr. In a control experiment, eight explants were exposed to the 98% N_2/2% O_2 gas mixture without $^1O_2$ for 2 hr. The explants were examined identically following completion of the control exposures.

Finally, we determined the effect of $^1O_2$ exposure on tracheal epithelium by exposing eight explants to 410 ppb $^1O_2$ for 2, 4, and 8 hr in separate experiments. Control experiments were also performed for each of the exposure periods. All ring explants were examined immediately after exposure.

Examination of Tracheal Cultures

Of the 24 tracheal explants exposed per $^1O_2$ concentration, 8 explants were used for ciliary activity measurements and cytological observations (*11*). Cilia beating frequency at the periphery of the mucosa of each ring was determined using a Type 1531-AB electronic stroboscope (General Radio, Concord, MA) as the light source and a Nikon inverted microscope. Beating frequencies were measured at each quadrant of the lumen and the mean recorded as beats per minute. The percentage of normal respiratory epithelium, i.e., a smooth luminal surface with beating cilia, was also determined for each tracheal ring. Focal ciliostasis was defined as

*Since the initial exposure study (*4*) we have observed a variation of $^1O_2$ concentration with gas flow rate through the $^1O_2$ generator. Consequently, preliminary studies showing the cytotoxic effects of $^1O_2$ were performed at a concentration of 827 ppb instead of the 121 ppb reported.

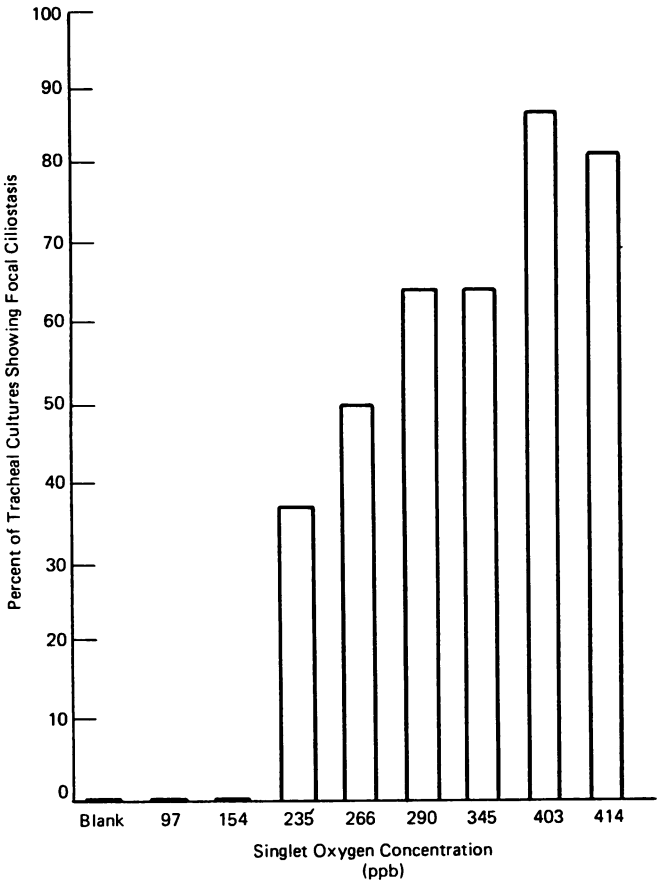

FIGURE 1. Effect of different singlet delta oxygen concentrations on the ability to produce focal ciliostasis in tracheal epithelium. Each bar represents the mean value of eight separate ring cultures.

no measurable ciliary motion at random areas on the luminal surface, as detectable by light microscopy.

Statistical Analysis

Data from studies designed to determine the immediate effects of $^1O_2$ and *in vitro* recovery were analyzed using two-way analysis of variance to test the hypothesis of no-treatment differences. Dunnett's test (*12*), a multiple comparison procedure for testing treatment means against a control mean, and the chi-square distribution test were used to determine patterns of significant treatment differences in cilia beating frequency and cytopathology. In statistical comparisons we chose $p < 0.05$ probability level for accepting or rejecting the significance of the differences between groups.

Results

Organ culture studies that involve measurements of ciliar activity require tracheal rings with healthy ciliated epithelium. By allowing the cultures to stabilize *in vitro* for 48 hr prior to exposure to $^1O_2$, we were able to minimize nonspecific damage. The tracheal ring explant system described in this work functioned well and gave consistent and repeatable responses in the background (before exposure) and control (exposure to the flowing gas stream without $^1O_2$) experiments. The data presented in Table 1 show that tracheal rings exposed to concentrations of 154 to 414 ppb $^1O_2$ exhibited a significant decrease in cilia beating frequency. At concentrations as low as 154 ppb, $^1O_2$ produced a dose-dependent decrease in ciliary activity. At 414 ppb $^1O_2$, we observed a 35% decrease from the background measurement and from the control postexposure values. Focal ciliostasis also increased with increased $^1O_2$ concentrations. Cultures exposed to $^1O_2$ at 266 ppb or greater produced 50 to 88% focal ciliostasis (Fig. 1).

The appearance of the epithelium from explants exposed to $^1O_2$ was different from the control (Table 1). Observations made on eight separate explants with phase optics showed sloughing of epithelial cells, as well as cells protruding into the lumen. The extent of these cytological changes depended on the concentration of $^1O_2$ and were first observed at a concentration of 235 ppb.

The time required for the tracheal explants to recover from a 2-hr exposure to 401 ppb $^1O_2$ was studied (Table 2). Observations made at 1 and 2 hr after exposure still showed a statistically significant reduction in cilia beating frequency and cytological alterations. None of the cultures showed focal ciliostasis after 1 hr recovery. After 4 hr recovery, the mean cilia beating frequency (1163) is significantly different than the control value (1197), and the mean percentage of normal epithelium is at 79%, which is also statistically different from the control value (87%). By 8 hr, both the ciliary activity and cytological observations approached the normal ranges.

Table 3 shows the effects of 2-, 4-, and 8-hr exposure of tracheal rings to an $^1O_2$ concentration of 410 ppb. Significant changes in ciliary activity and cytopathology were observed after 2 hr exposure. At the end of 4-hr exposure, the values for both the mean cilia beating frequency and the mean percent normal epithelium continued to decrease. The 8-hr exposure values show a cilia beating frequency of 663 beats/min with 40% of the normal epithelium remaining. These values were not significantly different from the 4-hr treatment. No significant changes were observed in separate control experiments conducted for 2, 4, and 8 hr.

Discussion

Hamster tracheal organ cultures have been used extensively to study *in vitro* effects of environmental agents on mucociliary activity (*4,13–18*). Results with this model system have shown a close correlation with *in vivo* studies (*17,18*), suggesting that hamster tracheal epithelium is an excellent model for measuring the effects of inhaled environmental agents on the upper respiratory tract. The system and methods for treating tracheal explants with gas-phase $^1O_2$ appeared to func-

Table 2. Relationship of time to recovery from cytological alterations and decreased ciliary activity of *in vitro* hamster tracheal rings exposed to 401 ppb $^1O_2$.[a]

Treatment	Time, hr	Mean cilia beating frequency, beats/min				Mean % normal epithelium				% Of cultures showing focal ciliostasis
		Background	SE	Post-exposure	SE	Background	SE	Post-exposure	SE	
Exposure										
Control	2	1245	4	1197	7	95	3	89	2	0
$^1O_2$	2	1236	4	753*	62	94	1	48*	2	100
Recovery										
—	1[b]	—	—	1044*	94	—	—	63*	4	0
—	2[b]	—	—	1103*	8	—	—	71*	2	0
—	4[b]	—	—	1163	5	—	—	79*	2	0
—	8[b]	—	—	1178	5	—	—	83	2	0

[a] Values represent means ± SE from eight separate ring cultures.
[b] Recovery time after completion of a 2-hr exposure period.
* Significantly different from 0-time baseline values and the blank postexposure ($p < 0.05$; χ^2 distribution test for percent normal epithelium, $p < 0.05$; Dunnett's test for beating frequency).

Table 3. Effects of various exposure times to approximately 400 ppb $^1O_2$ on tracheal epithelium *in vitro*.[a]

Treatment	Time, hr	Mean cilia beating frequency, beats/min				Mean % normal epithelium				% Of cultures showing focal ciliostasis
		Background	SE	Post-exposure	SE	Background	SE	Post-exposure	SE	
Control	2	1242	5	1200	4	96	1	89	2	0
$^1O_2$ 414ppb	2	1215	5	793*	90	91	2	58*	7	75
Control	4	1234	4	1168	4	93	1	84	2	0
$^1O_2$ 416 ppb	4	1224	4	674*	63	93	1	42*	8	100[b]
Control	8	1229	4	1180	4	94	1	85	1	0
$^1O_2$ 401 ppb	8	1223	3	663*	68	91	1	40*	7	100[b]

[a] Values represent mean ± SE from eight separate ring cultures.
[b] All $^1O_2$ exposed cultures (100%) showed random areas of focal ciliostasis.
* Significantly different from 0-time baselines values and the blank postexposure ($p < 0.05$; χ^2 distribution test for percent normal epithelium, $p < 0.05$; Dunnett's test for beating frequency).

tion well and gave consistent results in both test and control cultures.

The results obtained in the present study show that gas-phase $^1O_2$ produces a significant decrease in ciliary activity and that higher concentrations give a more pronounced response. The epithelium of $^1O_2$-treated explants was moderately affected, and the percentage of tissue affected increased with increasing $^1O_2$ concentrations. At an $^1O_2$ concentration of 414 ppb, focal ciliostasis, i.e., no measurable ciliary movement at various sites along the lumen, was also pronounced. When cultures were allowed to recover in maintenance medium following exposure to a concentration of 410 ppb $^1O_2$, ciliary activity increased gradually with time. After 4 hr, the beating frequency of the cultures exposed to $^1O_2$ was indistinguishable from control cultures.

At the present time, it is impossible to determine whether singlet oxygen or a secondary toxic oxygen species, presumably a free radical, is responsible for the observed toxic effects. Studies in our laboratory with model membrane systems indicate that $^1O_2$ reacts with phospholipids to form hydroperoxides. These hydroperoxides could, in turn, decompose to give free radicals. We are planning experiments to determine whether $^1O_2$ can pass through a model membrane and thus potentially be responsible for the observed effects. The involvement of other toxic oxygen species will also be investigated. In addition, we plan to identify intracellular sites of damage such as the mitochondria by looking at enzyme inactivation and cellular ATP levels.

The development of methods for the generation of and exposure to $^1O_2$ has led to significant advances in demonstrating the action of $^1O_2$ on the respiratory epithelium. The clearly observed concentration-dependent response of ciliary activity along the concentration range studied is evidence that the *in vitro* system we used is sensitive for measuring the effects of $^1O_2$ on mucociliary respiratory epithelium.

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