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Increased 8-Hydroxyguanine Content of Chloroplast DNA from Ozone-Treated Plants[1]

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ABSTRACT

The mechanism of ozone-mediated plant injury is not known but has been postulated to involve oxygen free radicals. Hydroxyl free radicals react with DNA causing formation of many products, one of which is 8-hydroxyguanine. By using high performance liquid chromatography with electrochemical detection, the 8-hydroxy-2′-deoxyguanosine (8-OHdG) content of a DNA enzymatic digest can be sensitively quantitated. Beans (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.) were treated with an ozone regime that caused acute injury. Chloroplast DNA was obtained from plants harvested either immediately after ozone treatment or 24 hours later. Ozone-exposed plants in general had nearly two-fold higher levels of 8-OHdG as compared to control plants. *In vitro* treatment of DNA in buffer solution with ozone did not cause formation of 8-OHdG in DNA, even though ozone did react directly with the macromolecule *per se*. Exposure of isolated, illuminated chloroplasts to ozone caused nearly a seven-fold increase in the amount of 8-OHdG in the chloroplast DNA as compared to none-ozone-exposed chloroplasts. These results suggest that ozone exposure to plants causes formation of enhanced levels of oxygen free radicals, thus mediating formation of 8-OHdG in chloroplast DNA. The reaction of ozone with DNA *per se* did not cause formation of 8-OHdG. Therefore, it is the interaction of ozone with plant cells and isolated chloroplasts which mediates oxygen free radical formation.

Ozone is one of the most potent widespread phytotoxins known (14). It has been implicated as a key agent which, in part, may be responsible for the tree decline observed in Europe and in parts of the United States and Canada (2, 11, 19, 21, 26). Ozone-mediated plant injury has been investigated for some time, yet no clear-cut mechanisms have emerged.

The involvement of oxygen free radicals has been implicated in ozone-mediated plant injury (22, 26), yet direct proof has been difficult to obtain due to the lack of methodology to quantitate the normally extremely low levels of oxygen free radicals *in vivo*. Grimes *et al.* (12) demonstrated that ozone exposure to aqueous solutions containing the spin-trap, dimethylpyrroline-*N*-oxide, yielded a hydroxyl free radical adduct. We have developed a new method to quantitate oxygen free radicals in biological systems (6–9). The approach depends upon HPLC with electrochemical detection of products formed as a result of their reaction with oxygen free radicals, specifically hydroxyl free radicals. This approach has been used with either an exogenous hydroxyl free radical trap, salicylate (6, 8), or an endogenous macromolecule, DNA (7, 9, 10).

Hydroxyl free radicals react with DNA to yield numerous products; one is guanine, which becomes hydroxylated at the 8-carbon position (10, 16). Following hydroxyl free radical reaction with DNA and its digestion to the nucleoside, 8-OHdG[2] (7, 10, 16) can then be directly quantitated by LCED. The detection sensitivity with LCED is 20 fmol (7). With DNA as an indicator of hydroxyl free radical occurrence and LCED methodology, it was possible to ascertain that oxidative damage to DNA had occurred in a living system subjected to oxidative stress (7). Kasai and colleagues (15) have also demonstrated the enhanced formation of 8-OHdG in DNA of cells subjected to oxidative stress. A direct demonstration of the increased level of oxygen free radicals in ozone-treated plants may help understand the mechanisms involved in the damage. The results presented here support the hypothesis that ozone treatment induces an increased level of oxygen free radical flux *in vivo*. Since the presence of 8-OHdG in the DNA template has been shown to cause misreading of DNA (18), it is possible that 8-OHdG in chloroplast DNA of ozone-treated plants may amplify the damage by causing errors in protein synthesis or DNA replication.

MATERIALS AND METHODS

Plant Culture

Peas (*Pisum sativum* L. cv Alsweet) and beans (*Phaseolus vulgaris* L. cv Blue lake 290) were grown from untreated seeds in an artificial medium (Promix BX[3]) and watered with a complete nutrient solution as described previously (23). The plants were cultured in controlled environment chambers (Conviron model E15) at light/dark air temperatures and RH of 21/20°C and 75/90%, respectively, and a photosynthetic

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[2] Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; LCED, high performance liquid chromatography-electrochemical detection.

[3] Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the U.S. Environmental Protection Agency.

photon flux density of 300 μmol m^{-2} s^{-1} (at canopy height) for 16 h/d as previously described (23).

Ozone Exposure

Plants 21 d old were exposed in small exposure chambers housed in growth chambers (23). Environmental conditions were the same as the preexposure plant growth conditions. Ozone was generated by passing air over a UV light source and monitored by UV photometers (Dasibi model 1003 AH). The photometers were calibrated with a transfer standard and subjected to periodic zero and span checks to ensure accuracy (23).

Chloroplast Isolation

Chloroplasts were isolated from batches of leaves (25–50 g) collected from control and ozone-treated plants. Chloroplasts were isolated, in most cases, by the technique described by Lebherz *et al.* (20). To determine if the isolation method influenced results, chloroplasts were isolated by a sucrose gradient technique developed by Palmer (24) or by a Percoll gradient technique described by Cline *et al.* (5). Results obtained on DNA isolated in reference to Chl content and 8-OHdG content of the DNA indicated that the values were similar from all three methods.

DNA Isolation and Digestion

DNA was usually isolated by a procedure described by Gupta *et al.* (13), adapted to chloroplasts rather than animal tissue. This procedure involves the use of proteinase K and RNase digestion followed by chloroform/phenol/isoamyl alcohol (49:50:1) extractions. Care was exercised to ensure that the highest quality phenol (Aldrich Chemical Co., Gold Label) was used. For initial experiments, DNA was isolated by hydroxyapatite chromatography (1).

The isolated DNA was dissolved at 200 to 500 μg/mL and the *A* determined at 230 nm, 260 nm, and 280 nm. The *A* ratios of 260/280 nm and 230/260 nm were found usually to be more than 2:1 and less than 0.8, respectively. The DNA was dissolved in 5 mM Bis-Tris/0.1 mM EDTA buffer (pH 7.1) and digested to the nucleoside level according to the procedure described by Beland *et al.* (1).

8-OHdG Determination

The 8-OHdG concentration in the DNA digest was determined as described previously (7, 9, 10) by LCED. The 8-OHdG standard was synthesized and characterized by the method of Kasai and Nishimura (16) as described previously (9).

Chloroplast Exposure to Ozone and Light

Chloroplasts were isolated from 21-d-old beans according to the procedure of Lebherz *et al.* (20). Chloroplasts, 6 g fresh weight, were suspended in 40 mL of a medium consisting of 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes buffer (pH 7.6). One-half of the suspended chloroplasts was used for ozone treatment, and the other one-half was used for controls exposed to charcoal-filtered air. The chloroplast solution (20 mL) was placed in a glass dish to a depth of 0.6 cm. The glass dish was placed in a 10°C water bath and gently stirred with a magnetic stirrer. Chloroplasts were illuminated by placing a desk lamp with two 15-w fluorescent bulbs within 30 cm of the solution surface. Either 4.5 L/h ozone-enriched air (400 ppb) or charcoal-filtered air was flowed at a rate of 4.5 L/h and bubbled into the chloroplast solution. After treatment for 1 h, the DNA was isolated from the chloroplast solutions according to Gupta *et al.* (13).

DNA Exposure to Ozone

Calf thymus DNA was solubilized in 1.5 mM NaCl, 0.15 mM Na-citrate, 0.1 mM EDTA (pH 7.0), to a level of 250 μg/mL. The DNA was treated for 15 min by magnetically stirring it in a beaker where the solution surface was exposed to a stream of ozone (200 ppb) or charcoal filtered air at 4.5 L/h under subdued light. The *A* of the DNA at 260 nm was determined, and the DNA was precipitated with ethanol and digested to the nucleoside level as described above.

RESULTS AND DISCUSSION

Ozone-induced injury was visible on bean and pea leaves within 24 h of exposure. The isolated chloroplast DNA from the exposed plants displayed a significant increase in the concentration of 8-OHdG (Table I). This result was consistent among a number of experiments. Although there was some variation, the ratio (ozone-exposed/control) of the 8-OHdG content was approximately 2:1 and was independent of the method used to isolate the DNA. One limitation of the data is that, for each treatment, plants were pooled to yield enough leaves for chloroplast DNA isolation and analysis. It was not possible, mainly because of exposure space and time restraints, for more than one batch of DNA to be obtained and analyzed for each treatment in an experiment. In many cases, a particular DNA digest was subjected to more than one HPLC analysis, in which case the average value is presented. Based on the data presented, it appears that the ratio of 8-OHdG content of chloroplast DNA from treated in relation to untreated plants was about 2:1 whether the plants were harvested immediately following exposure (0 h) or 24 h later. However, the results should not be interpreted as an indication of the lack of DNA repair. Rather, most likely the free radical processes involved in injury formation continue for some time after ozone exposure.

A marked increase in 8-OHdG concentration was also observed when isolated chloroplasts were illuminated while exposed to ozone (Table II). In the isolated chloroplasts, there was nearly a sevenfold increase in 8-OHdG. Given the different methods of ozone exposure, it is not possible to directly compare ozone concentrations within the cell of the intact plant with the ozone concentration impinging on the isolated chloroplasts. Consequently, the reasons for the different amount of 8-OHdG in chloroplast DNA are unknown but are probably related to the degree of oxidative stress to chloroplasts. Isolated calf thymus DNA solubilized in buffer was exposed to ozone to determine if the direct action of ozone on DNA caused formation of 8-OHdG within DNA. There

Table I. *8-Hydroxyguanine Content of Chloroplast DNA from Intact Plants Exposed to Ozone*

Plants	Ozone Treatment	Time after Treatment[a]	DNA Amount	8-OHdG	Ratio
		h	*μg*	*fmole/μg DNA*	
Bean	200 ppb/2 h	24	1600[b]	29.5	1.71
	Control	24	1600	17.3	
	300–500 ppb/3 h	24	426[b]	65.0	1.97
	Control	24	737	33.0	
	200–300 ppb/6 h, 2 d	24	809[b]	28.7	2.37
	Control	24	1905	12.1	
	200 ppb/3 h	0	82[c]	88.4	1.60
	Control	0	52	55.3	
	200 ppb/3 h	0	221[c]	58.3	2.64
	Control	0	319	22.1	
Pea	200 ppb/2 h	24	551[c]	6.3	1.97
	Control	24	913	3.2	

[a] Number of hours after ozone exposure when the leaf tissue was harvested. [b] Chloroplast DNA isolated by hydroxyapatite chromatography (1). [c] Chloroplast DNA isolated by method of Gupta *et al.* (13).

Table II. *8-Hydroxyguanine Content of Bean Chloroplast DNA from Isolated Chloroplasts Exposed to Ozone in the Light*

Treatment	DNA	8-OHdG	Ratio
	μg	*fmole/μg*	
400 ppb ozone 1 h—4.5 L/h	34.3[a]	439.5	6.87
Control filtered air 1 h—4.5 L/h	46.5	64.0	

[a] DNA was isolated by the method of Gupta *et al.* (13).

Table III. *Effect of Ozone on 8-OHdG Formation in Calf Thymus DNA*

Experiment Time	Ozone Treatment[a]	8-OHdG	$A_{260\,nm}$
min		*fmole/μg DNA*	
A-0	−	12	0.430
A-15	+	17	0.382
A-30	+	13	0.376
B-0	−	13	0.417
B-15	+	17	0.379
B-30	+	12	0.376

[a] Ozone, 200 ppb, flowing at 4.5 L/h while the DNA was stirred with a magnetic stir bar, temperature 4°C; +, with ozone; −, without ozone.

was a decrease in $A_{260\,nm}$ (Table III), indicating that ozone reacted directly with the DNA, as reported earlier (4). However, the exposure did not increase the concentration of 8-OHdG in the DNA. These data support the observation that the effects of ozone on chloroplast DNA are mediated through processes involving hydroxyl free radicals generated as a result of ozone action on the chloroplast *per se* rather than being a direct reaction of ozone with the chloroplast DNA.

The enhanced level of the hydroxyl free radical adduct, 8-OHdG, in chloroplast DNA of ozone-injured plants clearly indicates that ozone exposure caused events resulting in oxygen free radical-mediated oxidative damage to DNA. These data are direct evidence for the involvement of oxygen free radicals in ozone-mediated plant damage and extend previous observations (12, 22, 26).

The fact that ozone treatment of illuminated isolated chloroplasts caused a large increase in the 8-OHdG content of the chloroplast DNA certainly indicates that ozone action on the chloroplast *per se*, in the presence of light, produced oxygen free radicals that damaged DNA. In contrast, the direct action of ozone on isolated DNA did not result in 8-OHdG formation even though the ozone reacted with the DNA *per se* as evidenced by the decrease in $A_{260\,nm}$.

The enhanced level of 8-OHdG in chloroplast DNA as a result of ozone exposure, which has long been considered an oxidative stress, strengthens previous observations of enhanced 8-OHdG concentration in DNA of other biological systems exposed to oxidative stresses. For example, human granulocytes exposed to the tumor promoter tetradeconylphorbol-acetate (7), x-irradiation of mice (15), hydrogen peroxide treatment of bacteria (15), as well as carcinogen treatment of cells (17) all have been shown to enhance the 8-OHdG content of the DNA isolated from the treated organisms. The presence of 8-OHdG within DNA has been shown to change the fidelity of DNA transcription, not only at the base opposite 8-OHdG, but also up to two bases removed from the altered guanine (18). Thus, it is likely that the presence of 8-OHdG within DNA may have serious biological consequences. Even though there is considerable redundancy of DNA within the chloroplast (3), the presence of 8-OHdG would be expected to alter the gene products and may play a role in cellular death.

With regard to the amount of 8-OHdG in chloroplast DNA,

our data are the only information available now. However, a recent report by Richter *et al.* (25) is interesting in a comparative sense. They found that nuclear DNA of normal rat liver had 8-OHdG at a level of about 0.025 pmol/μg DNA, whereas mitochondrial DNA had 16 times more 8-OHdG (0.41 pmol/μg DNA). The values they obtained for mitochondrial DNA are more than we obtained for chloroplast DNA. However, we have found that the 8-OHdG content of beans was about 4 fmol/μg DNA, which is from one-fourth to one-eighth less than mitochondrial DNA. Interestingly, we noted in preliminary experiments that the 8-OHdG content of total DNA did not significantly increase after treatment of the beans with acute doses of ozone. Richter *et al.* (25) attributed the high levels of 8-OHdG in mitrochondrial DNA to an enhanced oxygen free radical flux in mitochondria compared with the nucleus. This may be the reason why the 8-OHdG content of chloroplast DNA is higher than that of the total DNA, of which only a small fraction is chloroplast DNA. This question, as well as other aspects including the range in 8-OHdG content between plant species, remains to be investigated.

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