Light-Mediated Conversion of Nitrogen Dioxide to Nitric Oxide by Carotenoids

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Plants are more susceptible to the toxic effects of nitrogen dioxide when exposure takes place in the dark. Beta-carotene and other common carotenoids react with nitrogen dioxide in the dark to yield intermediate nitrosating agents consistent with the formation of nitrite esters. Simultaneous exposure of carotenoids to NO_2 and light significantly reduced formation of nitrosating intermediates and resulted in the release of nitric oxide (NO) into the gas phase. Light-mediated reduction of NO_2 to NO by carotenoids may be an important mechanism for preventing damage in plants exposed to NO_2. The formation of nitrosating agents from the reaction of carotenoids with NO_2 suggests that their ability to prevent nitrosative damage associated with NO_2 exposure in both plants and animals may be limited in the absence of light. *Key words*: carotenoids, light-mediated reduction, nitric oxide, nitrogen dioxide, plant damage. *Environ Health Perspect* 102: 460–462 (1994)

Carotenoids are produced by plants to protect against oxidative and photolytic damage (*1*). Considerable indirect evidence suggests that dietary antioxidants may also provide protective functions in longer-lived animal species, including humans (*2,3*). Although the nature of oxidants acting in mammalian systems may in some respects differ from those in plants, the underlying mechanisms may be similar. In elucidating the mechanism(s) of NO_2 toxicity, as well as its mitigation by antioxidants, it is essential to consider both the direct and indirect toxicities manifested both by NO_2 and its reaction products.

The highly reactive NO_2 radical is formed from the oxidation of nitric oxide (NO), which is produced in high-temperature combustion processes, by bacteria or through normal biochemical processes in both plants and animals (*4–6*). Nitrogen dioxide damages living cells in a variety of ways, including oxidation of membrane lipids (*7*), *N*-nitrosamine formation (*8*), direct mutation of DNA (*9–11*), DNA single-strand breaks (*12*), and/or other oxidative reactions. Nitrogen dioxide accumulation in grain elevators is associated with "silo filler's disease" which is characterized by massive and often lethal lung damage in exposed individuals (*13*). The free radical reactions of NO_2 generally occur independently of light, while more stable species such as nitrite and metal nitrosyl complexes (e.g., nitroprusside) are converted, upon light exposure, to nitrosating species, such as NO^+ or nitrogen oxide radicals (*14–16*), which can subsequently react with nucleophiles to form nitroso compounds (*14,17*).

Exposure of plants to nitrogen dioxide in the dark causes acute injury and accumulation of nitrite, whereas simultaneous exposure to light reduces toxicity and nitrite formation (*18,19*). The mechanism of NO_2 toxicity in plants has not been elucidated, but likely involves both direct effects from exposure to this highly reactive free radical and indirect effects from the products formed when NO_2 reacts with cellular constituents. Although exposure to nitrogen oxides affects photosynthesis (*20*), it is not known if this is related to the acute toxicity of NO_2. Shimazaki et al. (*18*) have proposed that the accumulation of nitrite in the dark and subsequent light-mediated reaction of nitrite with chlorophyll accounts for the observed toxicity of NO_2. Such an explanation, however, does not provide a mechanistic basis to fully explain either the differential accumulation of nitrite in dark versus light-exposed plants or the manner by which simultaneous exposure to light reduces toxicity.

The high reactivity of carotenoids, found in many green plants, toward oxidants and free radicals suggests that carotenoids may play an important role in preventing free radical damage caused by NO_2 exposure. However, the reaction products of highly conjugated molecules, such as the carotenoids, with NO_2 have not been well characterized; consequently, the conditions under which carotenoids protect against NO_2 damage in plants or animals is unknown. Reaction of NO_2 with double bonds, such as those found in linoleic acid, or cholesterol, results in the formation of nitrosating agents, which have been identified as nitrite esters (*21*). Alpha-tocopherol, like cholesterol and linoleic acid, reacts with NO_2 to form a nitrosating agent, while γ-tocopherol, lacking a methyl group in the *ortho* position to the phenol, does not (*22*). Gamma-tocopherol is also more effective at preventing neoplastic transformation of cultured cells (*22*), a property that may be related to its unique reactivity with nitrogen oxides. Recently Bittrich et al. (*12*) reported that γ-tocopherol was the only tocopherol analogue able to significantly reduce single-strand DNA breaks in Chinese hamster lung fibroblasts exposed to NO_2. The protection afforded by γ-tocopherol may be related to its ability to reduce NO_2 to the more stable NO molecule without requiring light (*22*). Nitrosation of primary amines on DNA bases *in vivo* can result directly in genotoxic damage; therefore, formation of a nitrite ester, capable of nitrosating amines, could limit protection from nitrosative damage associated with NO_2 exposure. Conversion of NO_2 to NO may additionally protect against oxidative damage by way of the relative antioxidant properties of NO (*23*). To provide insight into the mechanism of dark-phase NO_2 toxicity, we used an *in vitro* model system to study the reactions of carotenoids with NO_2 and the effects of light on the products obtained.

As shown in Figure 1, β-carotene (10^{-5} M in hexane) reacted with NO_2 to produce NO in the presence of light, whereas control solutions in the dark showed no significant NO evolution. Measured NO levels in the gas phase exiting β-carotene solutions began to decrease significantly after 6–8 min of exposure to NO_2, corresponding to the observed decolorization of the carotenoid solution (Fig. 2) and destruction of the highly conjugated polyene system (color loss occurred independently of light indicating that the initial reaction between NO_2 and β-carotene was not light mediated). Higher concentrations of β-carotene (10^{-4} M) showed sustained NO emission during the 15-min exposure and were not completely decolorized over this time (data not shown). Although 8 min of exposure to NO_2 in the dark resulted in complete loss of color and the disappearance of all β-carotene as measured by HPLC, 24 hr later samples regained some color as well as a small amount of β-carotene in samples exposed less than 10 minutes (Fig. 2). Lutein, the major carotenoid found in dark green, leafy vegetables such as spinach, also reduced NO_2 to NO in the light (Fig. 3), as did lycopene, an acyclic carotenoid found in tomatoes, and canthaxanthin, a food colorant containing two keto groups (data not shown).

Carotenoid solutions exposed to NO_2 formed a nitrosating agent as evidenced by

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their ability to subsequently nitrosate morpholine (Fig. 4). Beta-carotene samples exposed to light during NO_2 treatment showed significantly less ability to nitrosate morpholine (54% decrease, p = 0.006 for the difference in means) as well as less Griess-Saltzmann reactive material (42% decrease, p = 0.008), indicating less nitrite and/or nitrite ester present. Lutein accumulated less nitrite/nitrite ester relative to β-carotene under both lighting conditions and showed a corresponding reduction in the ability to nitrosate morpholine (Fig. 4). Other carotenoids examined showed a similar pattern of nitrosation and light-mediated inhibition in spite of their structural differences, although the ability of lycopene exposed to NO_2 in the light to subsequently nitrosate morpholine was more variable for undetermined reasons (data not shown).

Carotenoids are lipophilic compounds that act as membrane antioxidants in plants to protect against free radical and oxidative damage (*1*). The double bonds in carotenoids make these compounds highly reactive toward singlet oxygen (*25*) or free radicals such as NO_2 (*11*). The tocopherols also prevent oxidative damage by reacting with radicals to form less reactive intermediates (*26*). Interestingly, γ-tocopherol is found primarily in seed oils, whereas chloroplasts contain α-tocopherol and carotenoids (*27*). Whether this differential localization in plant tissues is related to conditions of light and/or NO_2 exposure is unknown; however, it is consistent with the observed superiority of γ-tocopherol to reduce NO_2 to NO in the dark (*22*).

The light-mediated conversion of NO_2 to NO by plant antioxidants offers a plausible mechanism to explain decreased sensitivity to NO_2 toxicity in plants exposed to light. In addition to reducing the level of nitrite and/or nitrite esters formed and subsequent nitrosation reactions, the production of nitric oxide may provide additional antioxidant protection through its reaction with NO_2 and other intracellular free radicals to block radical chain propogation reactions. The reaction of NO with NO_2 to form N_2O_3 might prevent acute free radical damage from NO_2, although increasing delayed damage arising from the nitrosation of amines by N_2O_3, while the reaction of NO with lipid or oxygen radicals would result in chain termination. Such a mechanism may explain the superior ability of γ-tocopherol to prevent single-strand DNA breaks in cells exposed to NO_2 (*12*).

Insight into the chemical mechanism by which γ-tocopherol or illuminated carotenoids reduce NO_2 to NO is provided in a recent paper by Bosch and Kochi (*28*) in which they describe the reaction of

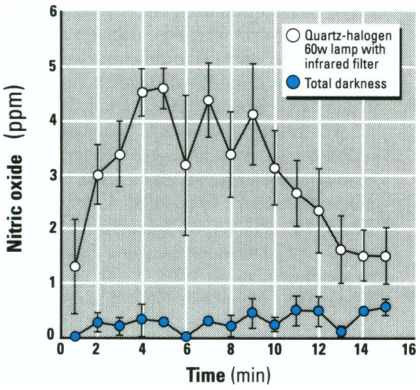

Figure 1. Effect of light on NO formation by NO_2-exposed carotene. Beta-carotene (10^{-5} M in 5 ml hexane) was exposed for the indicated times to 27.5 ppm NO_2 in N_2 by bubbling at a flow rate of 60 ml/min. NO was measured in the exit gas by direct injection of 10-µl gas samples into a thermal energy analyzer as previously described (*22*). Samples were kept in total darkness during exposure to NO_2 or placed 10 cm from a quartz-halogen 60-watt lamp with an intervening neutral density filter to block infrared light and prevent sample warming. Each point represents the mean of three separate determinations ± SE.

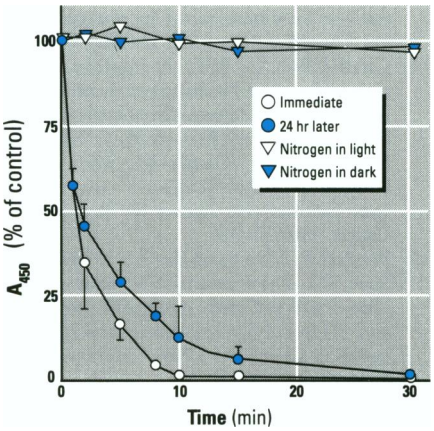

Figure 2. Change in A_{450} for β-carotene solutions exposed to NO_2. Samples of β-carotene (10^{-5} M in hexane), exposed as described in Figure 1 (n = 3) were measured immediately after exposure at the indicated times for absorbance at 450 nm (A_{450}). Samples that were remeasured 24 hr later showed some recovery of A_{450}, indicating that the loss was in part reversible. Control solutions of β-carotene in hexane bubbled (60 ml/min) with N_2 only in the dark or light are also plotted (n = 1). HPLC analysis (*22*) indicated a small quantity of β-carotene present at 24 hr versus none immediately after exposure to NO_2.

NO_2 with olefins to form NO and the corresponding epoxide. The formation of NO and an epoxide as opposed to nitrite ester formation depends on the nature of the olefinic compound as well as the reaction conditions employed. Recently Bors et al. (*29*) have shown that oxidative and genotoxic damage from 2-nitropropane *in vivo* is mediated through NO_2 formation and the direct reaction of NO_2 with DNA bases. Further work in this area is needed,

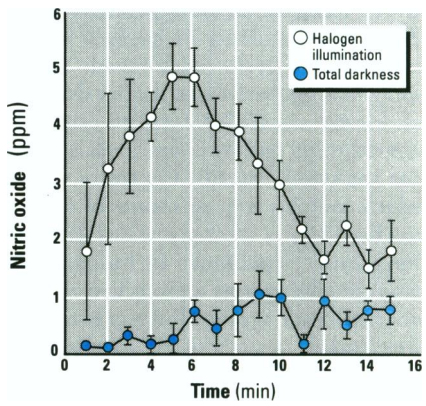

Figure 3. NO formation by lutein and NO_2 in the presence of light. Lutein (10^{-5} M in 5 ml hexane) was exposed for the indicated times to 27.5 ppm NO_2 in N_2 by bubbling at a flow rate of 60 ml/min and NO measured in the exit gas as described in Figure 1. During this period samples were maintained either in the dark or illuminated by a halogen light. Each point represents the mean of three separate determinations ± SE.

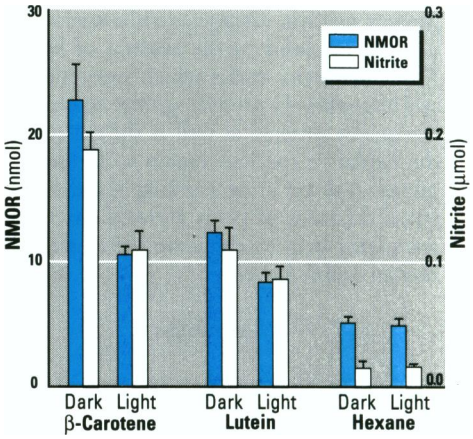

Figure 4. Nitrosation of morpholine and nitrite accumulation by carotenoids exposed to NO_2 in hexane. Nitrite accumulation and nitrosating potential of carotenoid or control solutions exposed to NO_2 for 15 min in either the dark or light as described in Figure 1 were measured. After NO_2 exposure was stopped, the volume was readjusted to 5 ml with hexane and an aliquot removed for nitrite analysis (*24*). Five minutes later, 50 µl of 100 mM morpholine and *N*-nitrosopyrolidine (200 µg/ml as internal standard) in hexane was added to the 5-ml sample. One milliliter of this solution was then placed into an amber vial and incubated at 37°C. A 1-µl sample was removed after 24 hr and analyzed by gas chromatography-thermal energy analysis for *N*-nitrosomorpholine as described previously (*22*). Data represent the mean of three separate experiments ± SE.

particularly with regard to the effect of light and neighboring substituents on the nature of products formed. Temperature may also play a role, particularly in animals, as we have observed increased rates of NO_2 reduction to NO by tocopherols at 37°C (data not shown).

Although the role of carotenoids in providing antioxidant protection in plants

is well accepted, an analogous function for dietary antioxidants in humans has not been conclusively established, despite numerous epidemiologic studies that show an inverse association between carotenoid consumption and/or serum levels and cancer incidence (*3,30*). Cigarette smoke, which contains large quantities of nitrogen oxides (*31*), is strongly associated with lung cancer incidence in humans, and many studies suggest a protective effect for antioxidants against lung cancer (*32*). Interestingly, a study of Fiji Islanders, who have extremely low lung cancer incidence rates, revealed only weak associations, negatively with carotenoids and positively with α-tocopherol, but did suggest a strong inverse association with plasma γ-tocopherol (*33*).

The observations described here offer a plausible explanation for the differential toxicity of NO_2 in plants exposed under various lighting conditions and suggest limitations in the ability of carotenoids to protect against nitrogen oxide-mediated damage in plants in the absence of light. The conditions under which carotenoids and tocopherols protect against oxidative damage in animals, as well as the nature of the oxidative species, remain to be determined. A better understanding of the dark-phase reactions of plant antioxidants may ultimately help to elucidate their roles in human health as well.

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