

Protection of Peroxynitrite-Induced DNA Damage by Dietary Antioxidants

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The present study was undertaken to test the hypothesis that dietary antioxidants protect DNA damage induced by peroxynitrite, a potent physiological inorganic toxin. The present study showed that dietary antioxidants such as (-)-epigallocatechin gallate, quercetin, rutin, resveratrol, and ursolic acid inhibit single strand breaks in supercoiled plasmid DNA induced by 3-morpholinodimethylamine N-ethylcarbamide (SIN-1), a generator of peroxynitrite through the reaction between nitric oxide and superoxide anion. The formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in calf thymus DNA by SIN-1 was also inhibited by dietary antioxidants. When U937 cells were incubated with 1 mM SIN-1 bolus, a significant increase of 8-OH-dG level was observed. However, oxidative DNA damage was significantly lower in the cells pre-treated with dietary antioxidants when cells were exposed to SIN-1.

Key words: Dietary antioxidants, Peroxynitrite, DNA damage

INTRODUCTION

Peroxynitrite (ONOO^-) anion is a potent oxidant generated from the interaction of nitric oxide (NO) and superoxide (O_2^-) (Beckman *et al.*, 1990). At physiological concentrations, NO is the only known biological molecule that can out-compete endogenous superoxide dismutase for available O_2^- (Beckman *et al.*, 1993) and formation of ONOO^- can count for both O_2^- and NO-dependent toxicities (Beckman *et al.*, 1990). The *in vivo* formation of this compound has been demonstrated in endothelial cells, neutrophils, neurons, macrophages and other cellular systems (Ischiropoulos *et al.*, 1992; Carreras *et al.*, 1994; Kooy and Royall, 1994). ONOO^- is a relatively stable species, but its protonated form decays with a rate constant of 1.3 s^{-1} at 25°C (Koppenol *et al.*, 1992). It can oxidize various biomolecules such as lipids, proteins, and thiols (Radi *et al.*, 1991). It also causes extensive base modifications as well as single-strand breaks in both supercoiled plasmid DNA and mammalian cellular DNA (Salgo and Pryor, 1996). Recent studies have implicated

ONOO^- in several inflammatory disorders and oxidative damage to DNA is one of the events taking place during chronic inflammation (Kaur and Halliwell, 1994).

Plant polyphenols are natural antioxidants and most of their pharmacological properties are considered to be due to their antioxidant action. This is generally considered to reflect their ability to scavenge endogenously generated oxygen radicals or those radicals formed by various xenobiotics (Ames *et al.*, 1995). The present study was undertaken to test the hypothesis that dietary antioxidants including polyphenols and triterpenoids such as (-)-epigallocatechin gallate (EGCG), quercetin, rutin, resveratrol, and ursolic acid directly protect from peroxynitrite-induced oxidative damage to DNA.

EGCG, one of the main catechins isolated from green tea, has been found to act as an effective antioxidant *in vitro* and *in vivo*. EGCG has thus been found to inhibit carcinogen-induced tumors in the skin, lung, duodenum, liver, and colon in rodents (Galati and O'Brien, 2004), by a mechanism believed to be related to the antioxidative properties. Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a major component of the polyphenols from grapes and red wine. This amphipathic molecule is capable of scavenging lipid hydroperoxyl free radicals as well as hydroxyl and superoxide anion radicals (Sun *et al.*, 2002). Ursolic acid, a pentacyclic triterpene acid, has been

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isolated from many kinds of medicinal plants, such as *Eriobotrya japonica*, *Rosmarinns officinalis*, and *Glechoma hederaceae*. Ursolic acid has been reported to produce antitumor activities and antioxidant activity (Balanehru and Nagarajan, 1991). Quercetin has been reported to be the most predominant flavonol and rutin as one of the most commonly found flavonol glycosides in the human diet. The flavonoids exhibit a wide variety of biological activities, including antiviral, antibacterial, anti-inflammatory, anti-carcinogenic, and antioxidant actions (Hertog *et al.*, 1993). Several studies have shown the flavonoids to act as scavengers of superoxide anions, singlet oxygen, hydroxyl radicals, and lipid peroxy radicals (Afanas'ev *et al.*, 1989).

The results demonstrate that dietary antioxidants exhibit the protective role in the peroxy-nitrite-mediated DNA damage reflected by the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) and single strand breaks.

MATERIALS AND METHODS

Materials

Calf thymus DNA, EGCG, quercetin, rutin, ursolic acid, resveratrol, and avidin-tetramethylrhodamine isothiocyanate (TRITC) conjugate were obtained from Sigma (St. Louis, MO, U.S.A.). 3-Morpholinolinosydnomine *N*-ethylcarbamide (SIN-1) were purchased from Calbiochem (La Jolla, CA, U.S.A.). pUC18 plasmid DNA was purified from *E. coli* cultures by using PROMEGA Magic Minipreps.

Cell culture

Human premonocytic U937 cells (American Type Culture Collection, Rockville, MD, U.S.A.) were grown in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, penicillin (50 units/mL), and 50 µg/mL streptomycin at 37°C in a 5% CO₂/95% air humidified incubator.

Strand breaks

DNA single strand breaks were assayed by measuring the conversion of covalently closed circular double-stranded supercoiled DNA (form I) to open relaxed circular double-stranded DNA (form II). pUC18 plasmid DNA (200 ng) was incubated in 25 mM phosphate buffer, pH 7.5 with a various concentrations of SIN-1 at 37°C for 3 h with or without each dietary antioxidant in a microfuge tube. The solution was immediately mixed after addition of SIN-1. The final volume was 10 µL. The reactions were terminated by the addition of 2 µL sixfold strength agarose gel loading solution (30% glycerol, 0.01% bromophenol blue). DNA samples were applied to 1% agarose gels in a TAE (40 mM Tris-acetate, pH 8.0/2 mM EDTA) buffer system, and electrophoresis was performed at 5 V/cm for 2 h at room temperature. Following electrophoresis, gels

were stained with ethidium bromide and then destained for several hours. DNA bands were visualized by illuminating the gel with UV light and photographed.

8-OH-dG level

Calf thymus DNA (200 µg/mL) in a 96-well plate was incubated with various concentrations of SIN-1 in 100 mM potassium phosphate buffer, pH 7.4 at 37°C for 2 h. After incubation, the DNA was precipitated by 1 M NaCl and ethanol. The DNA was dissolved in deionized water and reprecipitated with ethanol and washed several times to remove any remaining SIN-1. DNA was fixed with 4% (w/v) paraformaldehyde in PBS for 5 min at room temperature. After fixation the aqueous phase was removed by vacuum manifold. Nonspecific binding sites were blocked by incubating the DNA with 1% (w/v) gelatin in PBS for 5 min. The blocking solution was removed by vacuum manifold and the wells were washed 3 times with PBS. Avidin-TRITC conjugate was added to the wells (1:400 in PBS, 100 µL/well) and incubated at 37°C for 1 h in a humidified chamber. Following washing with PBS three times, detection of bound TRITC-labeled avidin was performed. The level of binding was quantified with a fluorescence plate reader with excitation at 540 nm and emission at 588 nm. 8-OH-dG levels of U937 cells were estimated by using a fluorescent binding assay as described by Struthers *et al.* (1998). After U937 cells were pre-treated with each dietary antioxidant (10 mM) for 2 h, cells were exposed to 1 mM SIN-1. After 18h cells were fixed and permeabilized with ice-cold methanol for 15 min. DNA damage was visualized with avidin-conjugated TRITC (1:200 dilution) for fluorescent microscope with 540 nm excitation and 588 nm emission.

Quantification of relative fluorescence

The averages of fluorescence intensity from fluorescence images were calculated as described (Sundaresan *et al.*, 1995).

Statistical analysis

The difference between two mean values were analyzed by Student's *t*-test and was considered to be statistically significant when $p < 0.05$.

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

RESULTS AND DISCUSSION

When pUC18 plasmid DNA was exposed to a bolus addition of SIN-1, the covalently closed circular double-

stranded supercoiled DNA (form I) was converted to a relaxed open circular DNA (form II) with single strand breaks (Fig. 1A) in a dose-dependent manner. SIN-1 produces NO and $O_2^{\cdot-}$ simultaneously in physiological solution at 37°C and the reaction between NO and $O_2^{\cdot-}$ leads to the formation of ONOO⁻. In the presence of EGCG, the extent of SIN-1-mediated DNA damage decreased in a dose-dependent manner (Fig. 1B). The dose-dependent protection of single strand breaks was also observed with resveratrol, ursolic acid, quercetin, and rutin, however, the protective effect was less pronounced with quercetin and rutin. One major consequence of the cellular formation of peroxynitrite would be the release of hydroxyl radical ($\cdot OH$) or $\cdot OH$ -like species upon decomposition (Beckman *et al.*, 1990). Results suggest that

dietary antioxidants play a role as a scavenger of ONOO⁻, presumably, through the removal of $\cdot OH$ or $\cdot OH$ -like species. Strand breaks are known indicator of hydroxyl radical attack on DNA (Park and Floyd, 1994).

The reaction of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) with DNA resulted in numerous forms of base damage, and 8-OH-dG is one of the most abundant and most studied lesions generated. Peroxynitrite reacts preferentially with guanine in DNA, and the majority of mutations caused by peroxynitrite occur at G:C base pairs (Juedes and Wogan, 1996). Because 8-OH-dG causes misreplication of DNA (Shibutani *et al.*, 1991), it has been implicated as a possible cause of mutation and cancer. Therefore, 8-OH-dG has been used as an indicator of oxidative DNA damage *in*

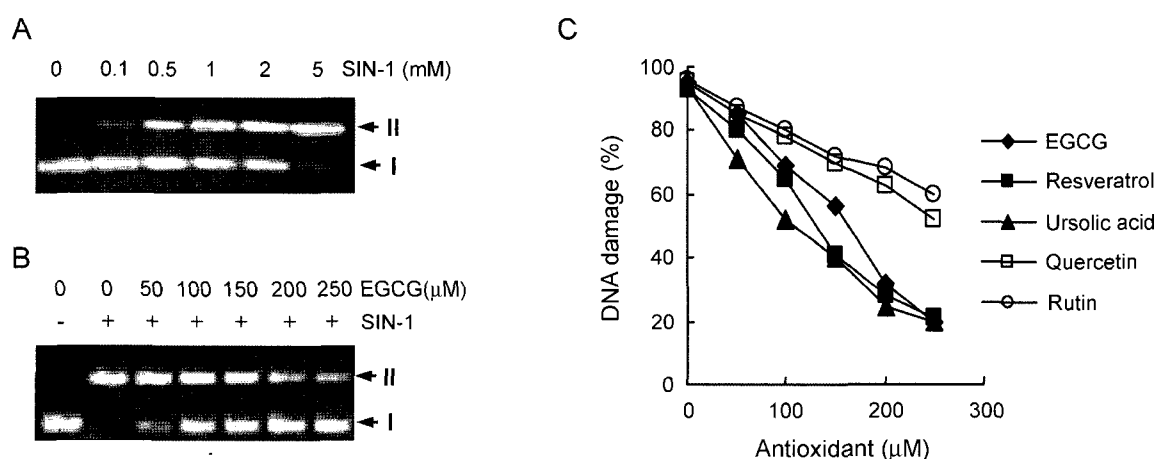


Fig. 1. Strand breaks in plasmid DNA induced by SIN-1 and its protection by dietary antioxidants. Supercoiled pUC18 plasmid DNA was incubated with SIN-1 for 3 h at 37°C. DNA damage is represented by the percentage of open circular (type II) DNA to the sum of open circular and supercoiled (type I) DNA. (A) Representative agarose gel indicating the concentration-dependent effect of SIN-1 on DNA. (B) The protection by different doses of EGCG against the strand breaks in plasmid DNA from 5 mM SIN-1 with 3-h exposure. (C) Quantitative analysis for the protection by different doses of dietary antioxidants against the strand breaks in plasmid DNA from 5 mM SIN-1 with 3-h exposure.

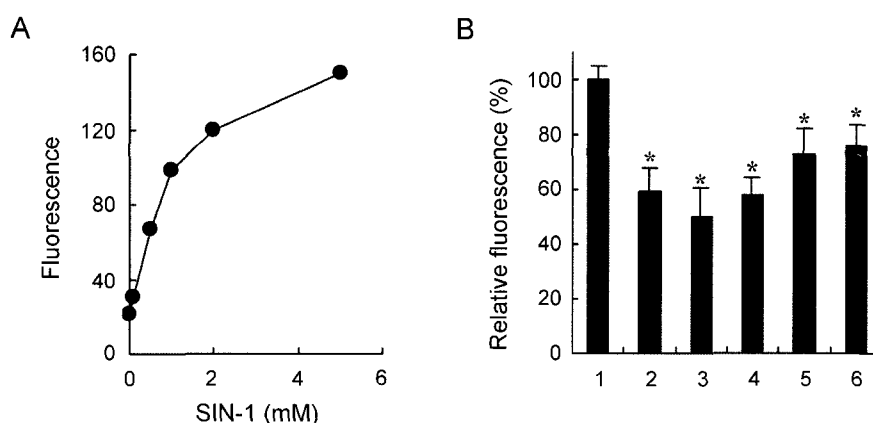


Fig. 2. Generation of 8-OH-dG in calf thymus DNA by peroxynitrite or SIN-1 and its protection by dietary antioxidants. (A) Concentration-dependent increase of 8-OH-dG in calf thymus DNA exposed to SIN-1 for 2 h at 37°C. (B) The protection by dietary antioxidants (150 μM each) against the generation of 8-OH-dG in calf thymus DNA from 5 mM SIN-1 with 2-h exposure. Values are means \pm S.D. of four determinations. 1, no antioxidant; 2, + EGCG; 3, + resveratrol; 4, + ursolic acid; 5, + quercetin; 6, + rutin. * $p < 0.05$ compared to control.

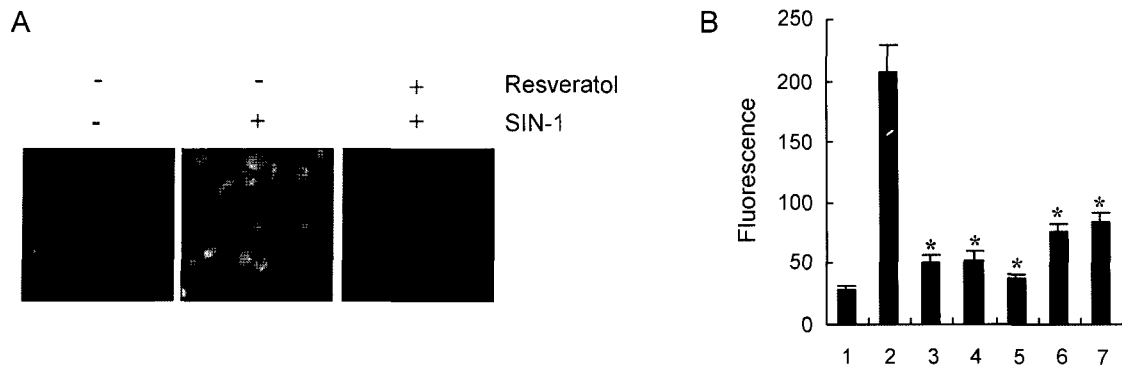


Fig. 3. The effect of dietary antioxidants on 8-OH-dG levels in U937 cells exposed to 1 mM SIN-1. (A) 8-OH-dG levels in U937 cells pre-treated with resveratrol (10 μ M) for 2 h upon exposure to SIN-1. 8-OH-dG levels reflected by the binding of avidin-TRITC were visualized by a fluorescence microscope. (B) The protection by different dietary antioxidants (10 μ M each) against the generation of 8-OH-dG in U937 cells exposed to SIN-1. The averages of fluorescence intensity were calculated as described (Sundaresan *et al.*, 1995). Values are means \pm S.D. of five determinations. 1, control; 2, + SIN-1; 3, + SIN-1 + EGCG; 4, + SIN-1 + resveratrol; 5, + SIN-1 + ursolic acid; 6, + SIN-1 + quercetin; 7, + SIN-1 + rutin. * $p < 0.01$ compared to control.

vivo and *in vitro* (Park and Floyd, 1992). Recently, it has been shown that 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC (Struthers *et al.*, 1998). The fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased in a concentration-dependent manner when calf thymus DNA was exposed to SIN-1 (Fig. 2A). Similar to the results obtained on strand breaks in plasmid DNA, 8-OH-dG formation in calf thymus DNA mediated by SIN-1 was significantly diminished by dietary antioxidants (Fig. 2B).

The fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased in untreated U937 cells upon exposure to SIN-1 (Fig. 3A). In contrast, the overall DNA appeared to be markedly protected in dietary antioxidant-treated cells even after exposure to SIN-1 (Fig. 3B). These results indicate that dietary antioxidants appear to protect cells from damage caused by peroxynitrite.

In conclusion, dietary antioxidants are effective in protecting DNA from damage caused by peroxynitrite, and alleviated damage suggests that further study of similar compounds is warranted.

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