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Antioxidative and Antimutagenic Activities of 4-Vinyl-2,6-dimethoxyphenol (Canolol) Isolated from Canola Oil

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A potent antioxidative compound in crude canola oil, canolol, was recently identified, and reported herein are studies of its scavenging capacity against the endogenous mutagen peroxynitrite ($ONOO^-$). $ONOO^-$ is generated by the reaction between superoxide anion radical and nitric oxide, both of which are produced by inflammatory leukocytes. Among various antioxidative substances of natural or synthetic origin, canolol was one of the most potent antimutagenic compounds when *Salmonella typhimurium* TA102 was used in the modified Ames test. Its potency was higher than that of flavonoids (e.g., rutin) and α-tocopherol and was equivalent to that of ebselen. Canolol suppressed $ONOO^-$-induced bactericidal action. It also reduced intracellular oxidative stress and apoptosis in human cancer SW480 cells when used at a concentration below 20 μM under H_2O_2-induced oxidative stress. In addition, canolol suppressed plasmid DNA (pUC19) strand breakage induced by $ONOO^-$, as revealed by agarose gel electrophoresis.

KEYWORDS: $ONOO^-$; mutagenicity; canolol; antioxidant; canola oil; radical scavenger; DNA breaks

INTRODUCTION

Most edible oils are usually obtained from plant seeds. Genomic conservation of these seeds is essential, so nutrients for development and germination of seeds must be protected from oxidative damage. However, the modern oil refining process aims to remove nonoil (or nonfat) components and other aromatic residues. Thus, edible oils today are mostly, if not completely, colorless and odorless. It is now known, however, that some components of these oils are important antioxidants for protection of nutritional compounds and genetic elements against oxidation, which affects lipidic and proteinous components as well as nucleic acids.

We studied edible oils from various sources and found that they differ with regard to scavenging capacity for lipid peroxyradicals ($LOO^•$) (*1*, *2*). $LOO^•$, formed from LOOH (lipid hydroperoxide; oxidized oils) in the presence of various heme components, exhibits a long half-life (>30 min) (*3*−*5*) and possesses a DNA-cleaving capacity (*1*, *2*, *6*, *7*). Free radical scavengers, or, more specifically, scavengers of $LOO^•$, in plant oils are important for the prevention of genomic damage within the seeds. Among vegetable and plant oils, extra virgin olive oil had the most potent $LOO^•$ -scavenging activity, followed by unpurified sesame oil and then unpurified canola oil (*1*). It is noteworthy that most commercial oils available in the market have very little $LOO^•$-scavenging capacity unless they are fortified with α-tocopherol. Most beneficial antioxidants are thus being removed from the oils as they are refined and become clear and odorless.

We have recently identified a potent antiradical component in pure form from crude canola (rapeseed) oil. The chemical structure was determined to be 4-vinyl-2,6-dimethoxyphenol, hereafter referred to as canolol (**Figure 1**). The concentration of canolol in crude canola oil was ∼200 ppm (*8*, *9*). After revision of this paper, a similar study was recently published by Koski et al. (*10*). In this study we have investigated the antimutagenic and antioxidative activities of canolol.

MATERIALS AND METHODS

Reagents. The nitric oxide (NO)-releasing agent 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (P-NONOate) was obtained from Dojindo Laboratories, Kumamoto, Japan.

Authentic peroxynitrite ($ONOO^-$) was synthesized from acidified $NaNO_2$ and H_2O_2 by a quenched-flow method according to the literature (*11*). The contaminant H_2O_2 was then decomposed by using manganese dioxide. The concentration of $ONOO^-$ was checked spectrophotometrically (*12*). All amino acids, taurine, rutin, protocatechuic acid, caffeine, ascorbic acid, α-tocopherol, sinapinic acid, $NaHCO_3$, and two known

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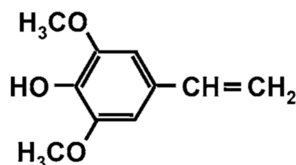

Figure 1. Structural formula of canolol. The chemical structure is 4-vinyl-2,6-dimethoxyphenol.

$ONOO^-$ scavengers, uric acid and ebselen, were obtained from Wako Pure Chemical Co. Ltd., Osaka, Japan. Nutrient broth and agarose were from Difco Laboratories, Detroit, MI. Heart infusion agar was from Nissui, Tokyo, Japan, and the minimal glucose agar plates were from Oriental Yeast Co., Ltd., Osaka, Japan. Dihydrorhodamine 123 and 2,7-dichlorodihydrofluorescein diacetate were from Molecular Probes, Eugene, OR. Canolol was originally obtained from crude canola oil, which was purified and characterized in our laboratories. Synthetic canolol was obtained from Showa Sangyo Co. Ltd., Funabashi, Chiba, Japan.

Bacteria and Culture Conditions. The following bacterial strains were generously provided by Dr. S. Arimoto, Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan: *Salmonella typhimurium* TA98 (TA98), to detect mutagens causing frameshifts; *S. typhimurium* TA100 (TA100) and *S. typhimurium* TA102 (TA102), to detect mutagens that cause base-pair substitutions (*13*). The TA102 strain is known to be sensitive to peroxides and aldehydes and undergoes mutation or revertant colony formation in the Ames test (*14*). We confirmed the histidine requirement, ampicillin resistance, and *rfa* mutation of these test strains (*13*). The efficiency of revertant colony formation by treatment with known mutagens was also confirmed as being within the normal range. Loopful aliquots of each test strain were inoculated into 5 mL of nutrient broth in L-shaped culture tubes, which were incubated at 37 °C for 20 h in a water bath with reciprocal shaking at 1 Hz. Cultured bacteria were washed three times with 0.85% saline by centrifugation (3000 rpm). This washing is required for the experiment on $ONOO^-$ exposure in the constant flux system, as described below.

Treatment with the Endogenous Mutagen $ONOO^-$, Mutagenicity Assay Using a Modified Ames Test, and Inhibition of Mutation. In this mutation assay system in contrast to that for the Ames test (*16*), no liver homogenate (supernatant), known as the S-9 mix, was used, because $ONOO^-$ does not require microsomal enzyme activation. The initial treatment used a bolus addition: mutagen, that is, 0.1 mL of $ONOO^-$ solution at specific concentrations, and 0.1-mL suspensions of *Salmonella* strains after 20 h of culture [$\sim 10^9$ colony-forming units (CFU)] were added to 0.5 mL of PBS, pH 7.4, and incubated for 20 min at 37 °C with reciprocal shaking at 1 Hz. A sample of the treated bacterial suspension was then added to 2 mL of soft agar containing 0.5 mM histidine and 0.5 mM biotin, and the mixture was poured onto a minimal glucose agar plate (63.6 cm^2). The solidified plates were incubated for 48 h at 37 °C in the dark. Revertant (i.e., mutant) colonies were counted by using a model CA-11 colony analyzer (System Science Co., Tokyo, Japan). In a control experiment, incubation was allowed to proceed (>10 min at neutral pH) to obtain a decomposed product of $ONOO^-$ in nutrient broth, and a 50-μL aliquot of decomposed $ONOO^-$ was placed in the test tube at 0 and 10 min.

The concentration of $ONOO^-$ used was 4 μM unless stated otherwise, which is pathophysiologically plausible. Because of the very short half-life of $ONOO^-$ at acidic to neutral pH (*17*), we used a constant flux system (**Figure 2**), in which the balance between influx and efflux (decomposition) was designed to maintain the 4 μM concentration or another specific concentration. PBS at pH 7.4 (0.52 mL) and a suspension of cultured bacteria (0.1 mL) were mixed gently by using a magnetic stirrer in a 2-mL vial. Then, $ONOO^-$ was constantly infused into the vial at a flow rate of 4 μL/min for 20 min by using a model ESP-64 automatic microsyringe pump (Eicom Corp., Tokyo, Japan). Concentrations of $ONOO^-$ used for the infusion were 7, 14, 21, and 28 mM, which resulted in final $ONOO^-$ concentrations of 2, 4, 6, and 8 μM, respectively, as determined spectroscopically. Each concentration of $ONOO^-$ was also measured by use of the dihydrorhodamine 123 oxidant assay, described elsewhere (*18*). Mutant colonies produced in the reaction mixture that grew on the minimal glucose agar plate were counted. The bactericidal activity of $ONOO^-$ was also measured by using the constant flux system of $ONOO^-$ and test bacteria.

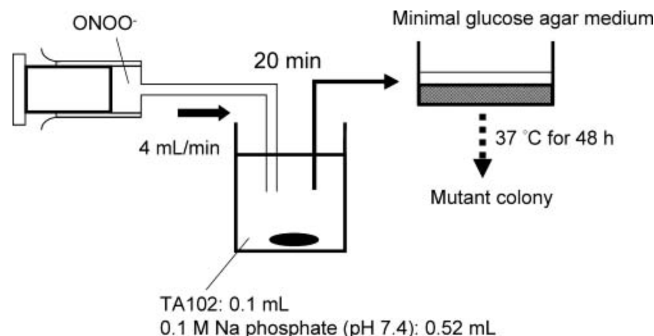

Figure 2. Diagram of the system used to ensure constant concentrations.

To examine the bactericidal activity of NO, an NO-generator, P-NONOate, which has an apparent half-life of 20 min at pH 7.0 and 37 °C, was added as a bolus to the bacterial suspension. Aliquots of the bacterial suspension were taken after incubations of 5, 10, and 20 min, diluted with nutrient broth, and seeded on the heart infusion agar plates. Numbers of bacteria were quantified by use of the colony-forming assay.

Suppression of mutant formation induced by $ONOO^-$ was investigated by adding uric acid, ebselen, various amino acids, rutin, protocatechuic acid, caffeine, sinapinic acid, ascorbic acid, α-tocopherol, $NaHCO_3$, or newly isolated canolol at various concentrations to the mutation assay system.

Antioxidant and Cytoprotective Activity of Canolol. Many cells reportedly undergo apoptosis under oxidative stress involving ROS (*19*, *20*). When human cancer SW480 cells were treated with either *tert*-butyl hydroperoxide (*t*-BuOOH) or pegylated zinc protoporphyrin (PEG-ZnPP), which are intracellular oxidative stress inducers, cells underwent apoptotic cell death. The possible protection afforded by canolol against this effect (oxystress) was examined in vitro. Cells were cultured as usual (10% fetal calf serum in Dulbecco's modified minimum essential medium under 5% CO_2/95% air at 37 °C), with or without oxidants, and were treated with various concentrations of canolol. Suppression of oxidative stress, as evidenced by induced fluorescence, was analyzed via the fluorescence-activated cell sorter (FACS) system, with 2,7-dichlorodihydrofluorescein diacetate as an indicator of intracellular oxidative stress (*19*, *20*). An assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was also performed, as described previously, to quantify the cytotoxicity or cytoprotective effect of canolol (*19*, *20*).

Oxidative DNA Breakage by $ONOO^-$ and Protection by Canolol. Under conditions similar to those for the bacterial mutation assay, *Escherichia coli* plasmid phage (pUC19) was subjected to treatment with 1−32 μM $ONOO^-$ in the constant flux system. Agarose gel electrophoresis was used to study canolol for its protective effect against DNA breaks, as described previously (*1*, *7*). Each DNA band was quantified by using the Atto Densitograph (Atto Co. Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Bactericidal Activity. The bactericidal activity of $ONOO^-$ was evaluated via the constant flux method and washed *S. typhimurium*. As shown in **Figure 3A**, bactericidal activity increased according to the exposure time and the concentration of $ONOO^-$. The survival rate of bacteria treated with 8 μM $ONOO^-$ for 20 min decreased dramatically from the initial 100% (1×10^8 CFU/mL) to 0.02% (2×10^4 CFU/mL). This result confirmed that the decrease in the number of revertant colonies at $ONOO^-$ concentrations of >4 μM was due to the bactericidal effect of $ONOO^-$ on surviving bacteria. The bactericidal system of $ONOO^-$ (various concentrations) was used to study the protective effect of canolol, added at various

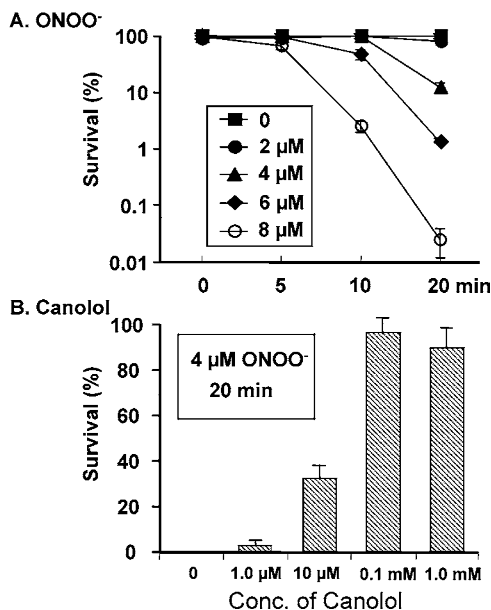

Figure 3. Bactericidal activity of $ONOO^-$ and canolol: (**A**) bactericidal activity of $ONOO^-$ at various concentrations measured in the constant flux system; (**B**) protective effect of canolol at various concentrations against the bactericidal activity of $ONOO^-$. Data from three independent experiments are expressed as means ± SD.

concentrations, and colonies formed were counted. **Figure 3B** indicates that canolol protected against the bactericidal activity of 4 μM $ONOO^-$. However, NO, given as the NO generator P-NONOate, showed no bactericidal activity up to a concentration of 1 mM given for 20 min (data not shown). No other agents except ebselen showed any bactericidal action within the range of concentrations used in this test; ebselen at a concentration of 0.1 mM killed 40% of the bacteria (data not shown).

Evaluation of Mutagenicity with a Modified Ames Test, without S9 Mix. The results of this mutagenicity test are shown in **Figure 4**. The number of mutant colonies was found constant regardless of the $ONOO^-$ concentration when bacteria were treated with $ONOO^-$ by a bolus manner (**Figure 4A**). This seems to result from the fact that almost all of the $ONOO^-$ decomposed instantaneously at neutral pH. Therefore, 50 μL of 10 mM $ONOO^-$ was added twice, at 0 and 10 min, in the same system. With this modification, the number of mutant colony from the TA102 strain clearly did increase (**Figure 3B**), whereas those of TA98 and TA100 did not. These results suggest that $ONOO^-$ caused base modification in TA102, the strain known to be sensitive to ROS (*14*). Bactericidal activity of $ONOO^-$ against the three strains was not seen at any of the given concentrations (**Figure 4C**).

In vivo, $ONOO^-$ is produced from O_2^- and $NO^\bullet$ at sites of infection and inflammation; the decomposition of $ONOO^-$ occurs simultaneously with production and continues for a prolonged time (days). For evaluation of the mutagenicity under conditions closest to the in vivo pathophysiological state, a constant flux method (**Figure 2**) was chosen to maintain a constant level of $ONOO^-$ (*21*). **Figure 5** shows results for mutant formation under these conditions, for an $ONOO^-$ concentration range from 0 to 8 μM, pH 7.4. For this mutagenicity assay, two different conditions, with and without nutrient broth, were examined. Results for washed and nonwashed TA102 bacteria differed (**Figure 5**). The number of mutant colonies of bacteria that had been washed three times in 0.85% saline decreased when concentrations of $ONOO^-$ were >4 μM. This decrease could be attributed to the bactericidal

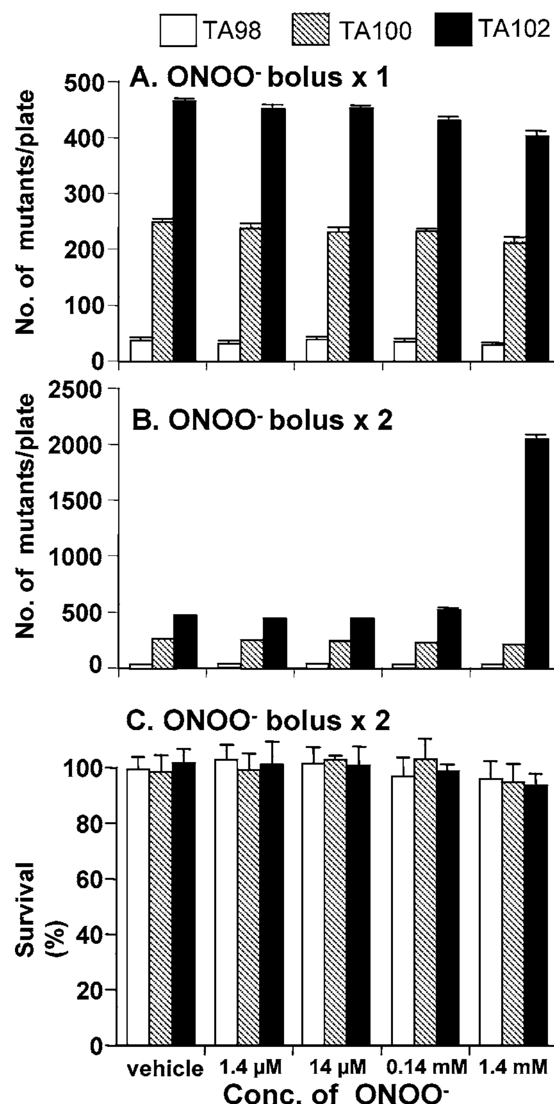

Figure 4. Evaluation of mutagenicity with a modified Ames test: (**A**) bacteria were treated with a bolus of 100 μL of 10 mM $ONOO^-$ added once; (**B**) in the same system, a bolus of 50 μL of 10 mM $ONOO^-$ was added twice, at 0 and 10 min; (**C**) bacterial survival after two bolus additions (at 0 and 10 min) of $ONOO^-$ at various concentrations. Data from three independent experiments are expressed as means ± SD.

activity of $ONOO^-$, as shown in **Figure 3A**. In the case of the nonwashed bacteria, that is, those cultured in nutrient broth, the number of mutants formed peaked at the concentration of 6 μM $ONOO^-$. The number of mutant colonies decreased at concentrations >6 μM $ONOO^-$ without nutrient broth (**Figure 5B**); more cells were killed at the higher concentration of $ONOO^-$. Decomposed $ONOO^-$ at the concentrations used caused no increase in the number of mutant colonies, which indicates that the decomposed $ONOO^-$ did not induce mutations.

Figure 5 also gives results for mutation frequency (right ordinate). The mutation frequency of washed bacteria increased according to the concentration of $ONOO^-$, up to 8 μM. At 8 μM $ONOO^-$, 21% of the total number of bacteria were mutant bacteria. This frequency is ~100000 times higher than that of control (spontaneous) mutation. The mutation frequency of nonwashed bacteria, however, was 3.8% at the concentration of 8 μM $ONOO^-$. This great difference is attributed to the fact that various scavengers in the medium, such as amino acids, nucleic acid moieties, and peptides, may react with $ONOO^-$ and suppress the mutation.

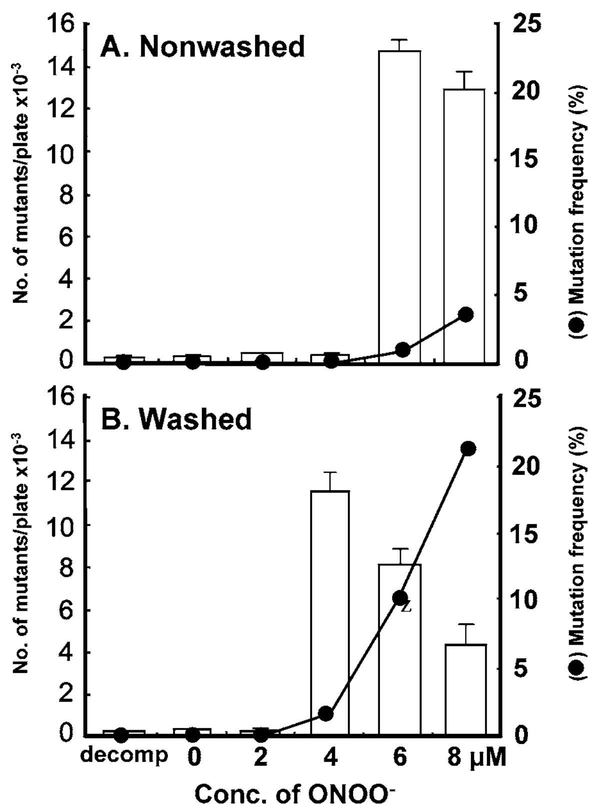

Figure 5. Formation of mutants and mutation frequency induced by $ONOO^-$ via the constant flux method. Two different types of TA102 bacteria were used: (**A**) nonwashed and (**B**) washed three times in 0.85% saline. Numbers of revertant colonies (mutants) and mutation frequency are shown by the bars and circles, respectively. The mutation frequency (percent) was calculated as the number of revertant colonies per the total number of viable colonies. Data from three independent experiments are expressed as means ± SD.

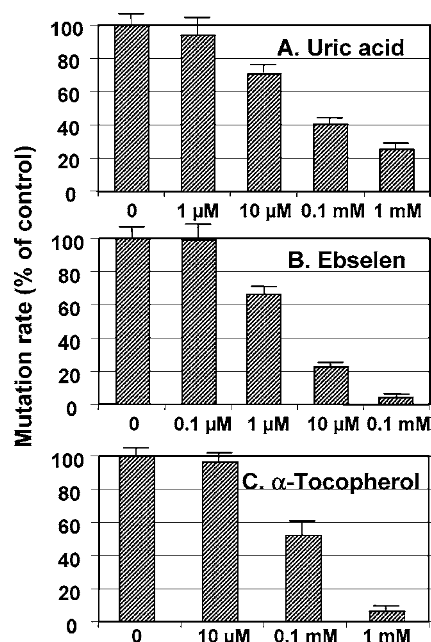

Figure 6. Antimutagenic effects of uric acid, ebselen, and α-tocopherol. Data from three independent experiments are expressed as means ± SD.

Antimutagenic Effects of Canolol and Other Compounds, including Uric Acid, Ebselen, Various Amino Acids, and Polyphenols. By means of the constant flux method, the effects of the typical $ONOO^-$ scavengers uric acid (*23*) and ebselen (*24*) on $ONOO^-$-induced mutagenesis were examined; scavenger concentrations ranged from 0.1 μM to 1 mM. Washed bacteria were used in this series of antimutagenic evaluations. Parts **A** and **B** of **Figure 6** show that uric acid at concentrations >10 μM and ebselen at concentrations >1 μM suppressed $ONOO^-$-induced mutation, in a concentration-dependent manner.

Because culture medium (nutrient broth) can itself suppress mutation induced by $ONOO^-$, we studied the antimutagenic effect of various amino acids, polyphenols, and other antioxidative substances and compared their potencies with that of canolol. **Figure 7** indicates that various compounds including amino acids and phenolic compounds reduced the mutation rate. L-Methionine reduced the mutation rate to 50% at 100 μM and to 16% at 1 mM; L-cysteine, to 65% at 100 μM and to 47% at 1 mM; taurine, to 64% at 1 mM (data not shown); and L-tryptophan, to 76% at 100 μM and to 17% at 1 mM. Both L-alanine and L-aspartic acid had almost no effect (99 and 92% at 1 mM, respectively; data not shown for either). Some plant-derived polyphenolic substances showed an antimutagenic effect; for example, rutin reduced the rate to 44% at 10 μM; caffeine, to 48% at 100 μM; and protocatechuic acid, to 45% at 1 mM. Ascorbic acid reduced the mutation rate to 87% at 100 μM and to 23% at 1 mM; $NaHCO_3$ (data not shown), to 82% at 100 μM and to 33% at 1 mM; and α-tocopherol, to 53% at 100 μM and to 7% at 1 mM. In previous studies, we found that bicarbonate (HCO_3^-) made $ONOO^-$ less reactive and that *Helicobactor pylori* showed better survival with the same concentration of $ONOO^-$ when bicarbonate was used, perhaps because this reactivity was extinguished when $ONOO^-$ reacted with various functional groups in the medium, such as amino acids, before the mutagen reached vital molecules in the bacteria (*12*, *21*). Other studies along this line have been published (*22*). In the present experiment, although bicarbonate reduced the mutagenic effect of $ONOO^-$, it was not as potent as canolol (**Figure 7I**) or α-tocopherol (**Figure 6C**). It was most interesting that sinapinic acid inhibited the mutation rate, to 43% at 10 μM and to 20% at 100 μM. Purified canolol, a decarboxylate moiety of sinapinic acid, exhibited a potent antimutagenic effect at concentrations >8 μM, in a dose-dependent manner (**Figure 7I**).

Antioxidative and Cytoprotective Effects of Canolol in Mammalian Cells. Human colon cancer SW480 cells were treated with two different oxidative stress-inducing agents. One was PEG-ZnPP, which inhibits heme oxygenase, thereby suppressing the intracellular generation of bilirubin, the antioxidative degradation product of heme, as described in recent publications (*19*, *20*, *25*). The other oxidative stress inducer was 25 μM *t*-BuOOH, which generates peroxyl radical after contact with heme or heme-containing compounds, including heme proteins. Canolol had antioxidative effects at a very low concentration (5.6 μM), with inhibition of PEG-ZnPP-induced ROS production, as revealed by FACS analysis (**Figure 8A**). Furthermore, the antiapoptotic effect of canolol was clearly demonstrated: it prevented apoptosis induced by oxidative stress caused by *t*-BuOOH (**Figure 8C**). Canolol alone at 560 μM was toxic to these cultured cancer cells in vitro (**Figure 8B**).

DNA Breaks Induced by $ONOO^-$ and Protection from Breakage by Canolol. The effect of exposure of supercoiled plasmid pUC19 DNA to $ONOO^-$ was investigated with the constant flux system. The closed circular form of DNA was converted to the single-nicked open circular form and the double-nicked linear form as a result of DNA strand breaks

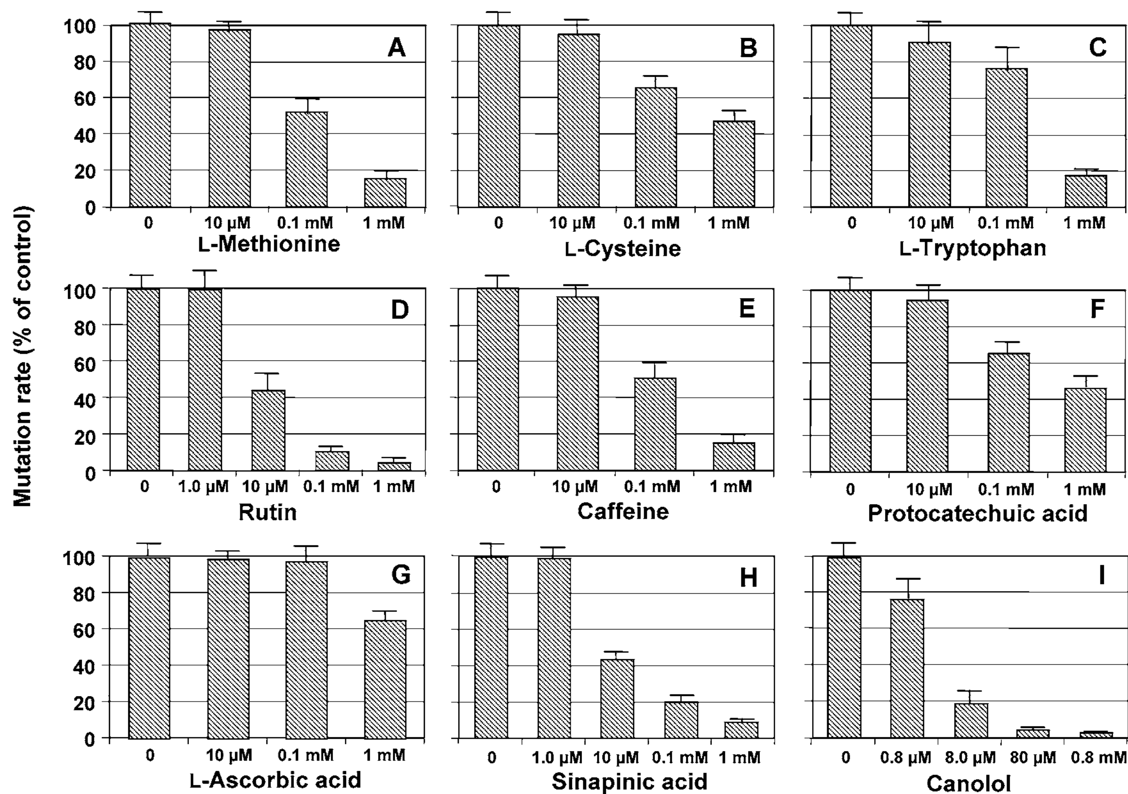

Figure 7. Antimutagenic effects of canolol, various amino acids, polyphenols, and other agents: (**A**) L-methionine; (**B**) L-cysteine; (**C**) L-tryptophan; (**D**) rutin; (**E**) caffeine; (**F**) protocatechuic acid; (**G**) ascorbic acid; (**H**) sinapinic acid; (**I**) canolol. Data from three independent experiments are expressed as means ± SD.

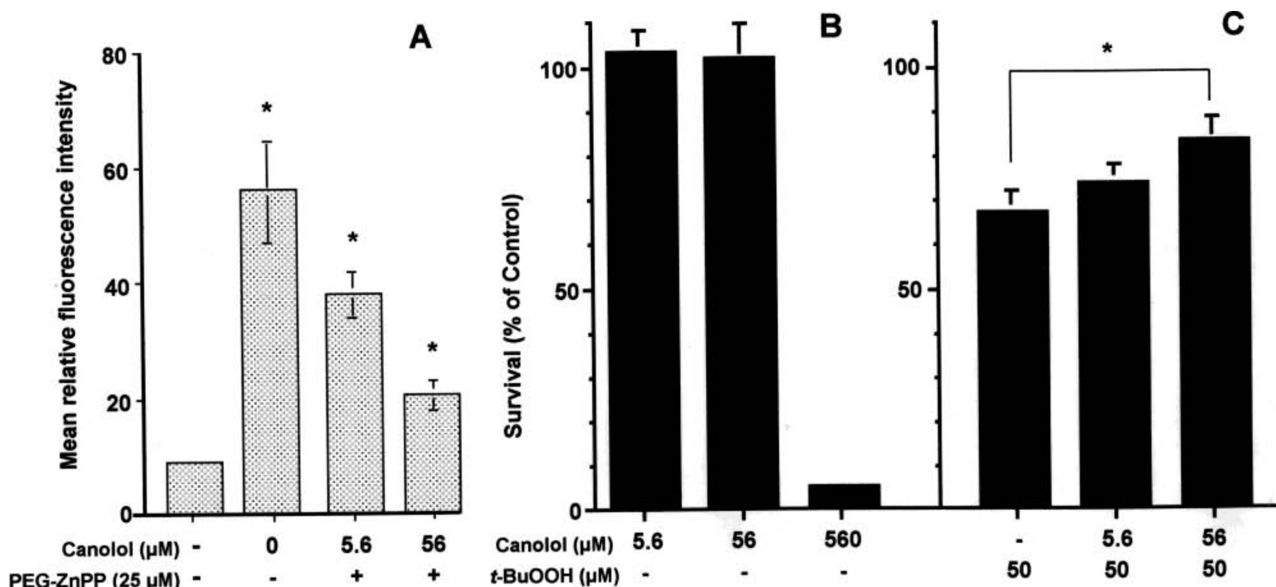

Figure 8. Antioxidative and cytoprotective effects of canolol in mammalian cells: (**A**) inhibition of PEG-ZnPP-induced intracellular ROS production by canolol [values are means ($n = 6$ wells); bars indicate SE; *, $p < 0.001$ versus untreated control]; (**B**, **C**) inhibition of *t*-BuOOH-induced cytotoxicity by canolol [values are means ($n = 6$ wells); bars indicate SE; *, $p < 0.05$].

induced by $ONOO^-$. As shown in **Figure 9A**,**B**, >80% of closed circular plasmid DNA became the open circular form in the presence of 1 or 4 μM $ONOO^-$, up to an $ONOO^-$ concentration of 8 μM (data not shown). Exposure to 16 or 32 μM $ONOO^-$ caused all DNA bands (closed circular, open circular, and linear forms) to disappear because of extensive oxidative degradation (data not shown).

The protective effect of canolol at concentrations ranging from 0.1 to 100 μM and 1 mM (not shown) was evaluated under the same conditions; the concentration of $ONOO^-$ was fixed at 1 or 4 μM (**Figure 9A**,**B**). The results showed that, in the presence of 1 μM $ONOO^-$, canolol prevented the formation of the open circular DNA when the concentration of canolol was >1 μM (**Figure 9C**). The decomposed products of $ONOO^-$ (applied at

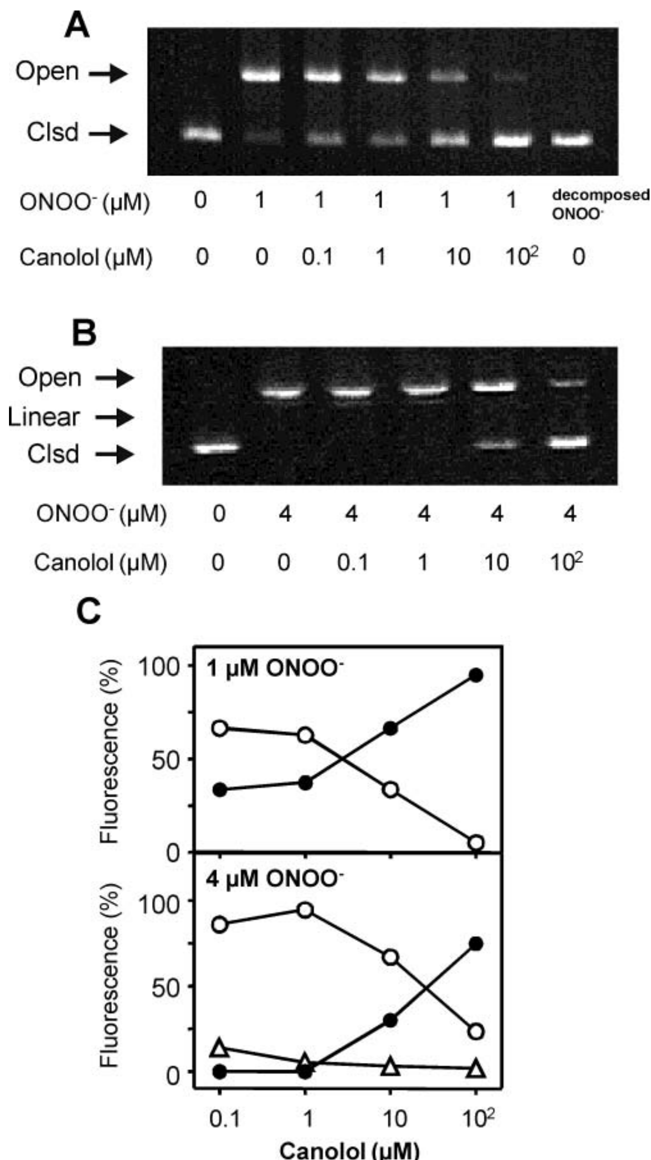

Figure 9. Protective effect of canolol against DNA breakage: (**A**) exposure of DNA to 1 μM $ONOO^-$ (final concentration) in the presence of various concentrations of canolol; (**B**) exposure of DNA to 4 μM $ONOO^-$ (final concentration) in the presence of various concentrations of canolol [Clsd and Open in (**A**) and (**B**) indicate closed circular and open circular DNA, respectively [cf. (**C**)]]; (**C**) quantification of fluorescence of DNA bands, as percentage of control (solid circles represent the closed circular form of DNA; open circles represent the open circular form; open triangles represent the linear form after double-strand breaks).

100 μM for 30 min) showed no effect on DNA, even when incubated for an additional 20 min (**Figure 9A**). These findings indicate that cleavage of plasmid DNA was induced by $ONOO^-$ at physiological concentrations and that canolol had a potent protective effect against DNA cleavage induced by $ONOO^-$.

$O_2^{\bullet -}$ and NO are representative free radical species generated in the body. They are generated by phagocytes at sites of inflammation, both react with each other rapidly at a diffusion rate-limited velocity, and they result in the formation of $ONOO^-$ (*15*, *26*, *27*). $ONOO^-$ is capable of efficient nitration and oxidation in vitro and in vivo, where targets of these chemical reactions are proteins, as evidenced by the production of nitrotyrosine, and nucleic acids (*12*, *28*−*31*). Also, $ONOO^-$ induces single-strand breaks of DNA (*32*). In this paper, we describe characteristics of a compound that we recently discovered in crude canola oil, namely, canolol, which has antimutagenic effects in bacteria and prevents $ONOO^-$-induced oxidative stress-related apoptosis of mammalian cells, as well as breaks of plasmid DNA strands (**Figures 8** and **9**).

NO is believed to be one of the most important defense molecules because of its bactericidal action, as reported by Hibbs et al. (*33*). However, NO itself is not bactericidal, whereas $ONOO^-$ has great potency as a bactericidal agent. The bactericidal principle derived from NO, shown in **Figure 3A**, and causing apoptosis in mammalian cells (**Figure 8**) is thus said to be $ONOO^-$. Canolol, at very low concentrations, offered protection against the bactericidal effect of $ONOO^-$ (**Figure 3B**). Canolol was also very effective in scavenging $LOO^\bullet$, generated in a system of hydroperoxides plus hemin, and protecting against $LOO^\bullet$-induced cell death (data not shown) (*1*, *3*).

In the evaluation of the mutagenic capacity of $ONOO^-$ in *Salmonella* strains used in the Ames test, strain TA102 showed the greatest effect, more than that of TA98 or TA100 (**Figure 4**). Therefore, strain TA102 was used in a modified Ames test (no S9 mix or liver homogenate was needed to activate promutagens). Because $ONOO^-$ has a very short half-life, <1 s at pH 7 (*26*), the frequency of $ONOO^-$-induced mutation did not depend on concentration when $ONOO^-$ was added as a single bolus; when two bolus additions were used, however, a higher mutagenic potential resulted at a concentration of 1.4 mM (**Figure 4B**), which suggests that the prolonged presence of this endogenous mutagen may be required. In fact, the constant flux system (**Figure 2**) that maintained a constant concentration of $ONOO^-$, ranging from 0 to 8 μM, a level equivalent to that found in pathological states in vivo, yielded a substantial mutation rate. Treatment of TA102 bacteria with 8 μM $ONOO^-$ for 20 min resulted in a mutation frequency of 21%; at a higher concentration of $ONOO^-$, the mutation rate increased, although the bactericidal effect was dominant (**Figures 3** and **5**).

Because nutrient broth had a protective effect against mutation, as shown by data for before and after washing with 0.85% saline (**Figure 5**), we investigated various amino acids and other antioxidant components of the culture medium that may react with $ONOO^-$ and hence suppress mutation. Two known $ONOO^-$ scavengers, uric acid and ebselen (*23*, *24*, *34*), were studied; they suppressed mutation, as expected (**Figure 6**). Among various amino acids and aromatic compounds, we found that thiol compounds (cysteine; data for glutathione not shown) and methylthiol compounds (methionine) had a suppressive effect against mutation (**Figure 7A**,**B**). Among flavonoids, rutin was very potent; it produced 50% suppression at $\sim$10 μM. Canolol showed the most potent inhibition against mutation of *S. typhimurium* strain TA102 of 18% at a concentration of 8 μM.

The anti-peroxyl radical activity of canolol was also examined with human colon cancer SW480 cells, as was protection against apoptotic cell death induced by treatment with *t*-BuOOH (*19*, *20*). Canolol at concentrations of 5.6−56 μM suppressed PEG-ZnPP (25 μM)-induced oxidative stress to a significant degree, as revealed by FACS analysis (**Figure 8A**). *t*-BuOOH at 50 μM caused $\sim$33% cell death; with 56 μM canolol, this apoptosis was suppressed 50% (**Figure 8C**). However, canolol at 560 μM was toxic by itself (**Figure 8B**).

Canolol also prevented, in a dose-dependent manner, DNA strand breakage induced by the endogenous oxidant $ONOO^-$ (**Figure 9**). This effect could be attributed to either an

antioxidative effect or termination of oxy radical-induced strand breaks such as the effect seen with malondialdehyde.

These data support the capacity of canolol to suppress $ONOO^-$-induced cell damage, killing of bacteria and mammalian cells, and bacterial mutation, as well as plasmid DNA strand breakage. Canolol also prevented oxidative stress-induced cellular apoptosis to a significant extent, although at the high dose of 100 μM it became cytotoxic to SW480 cells. These data suggest important biological and physiological roles for canolol and other antioxidants in plant seeds: protection of DNA and prevention of oxidation of lipids and proteins. These findings also have implications for the oil-refining process.

ABBREVIATIONS USED

FACS, fluorescence-activated cell sorter; ROS, reactive oxygen species; $ONOO^-$, peroxynitrite; $LOO^\bullet$, lipid peroxyl radical; $O_2^{\bullet -}$, superoxide anion radical; PEG-ZnPP, pegylated-zinc protoporphyrin; TA98, *Salmonella typhimurium* TA98; TA100, *Salmonella typhimurium* TA100; TA102, *Salmonella typhimurium* TA102; PBS, 0.1 M sodium phosphate-buffered 0.15 M saline, pH 7.4; NO, nitric oxide; P-NONOate, 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine.

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