Suppression of Hydroperoxide-induced Cytotoxicity by Polyphenols

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Abstract

A variety of synthetic and dietary polyphenols protect mammalian and bacterial cells from cytotoxicity induced by hydroperoxides, especially hydrogen peroxide (H2O2). Cytotoxicity of H2O2 on Chinese hamster V79 cells was assessed with a colony formation assay. Cytotoxicity and mutagenicity of H2O2 on Salmonella TA104 were assessed with the Ames test. SOS response induced by H2O2 was investigated in the SOS chromotest with Escherichia coli PQ37. The polyphenol-bearing o-dihydroxy (catechol) structure, i.e., nordihydroguaiaretic acid, caffeic acid ester, gallic acid ester, quercetin, and catechin, were effective for suppression of H2O2-induced cytotoxicity in these assay systems. In contrast, neither ferulic acid ester-bearing o-methoxyphenol structure nor a-tocopherol was effective, indicating that o-dihydroxy or its equivalent structure in flavonoids is essential for the protection. There are many reports describing that polyphenols act as prooxidants in the presence of metal ions. Our results suggest, however, that they act as antioxidants in the cells, when no metal ions are added to the medium.

Introduction

Hydroperoxides such as H2O2 and lipid hydroperoxides have been implicated as mediators of cellular injuries in a variety of clinical conditions including cancer. In particular, there are many reports describing the cytotoxic effects of H2O2 on mammalian cells, including viability, DNA lesions, mutation, chromosomal aberration, and morphological transformation (1-7). Occurrence of such oxidative cellular injuries itself indicates deficiency of cellular antioxidants such as a-tocopherol, ascorbic acid, glutathione, catalase, superoxide dismutase, and glutathione peroxidase in the peculiar conditions. One possible way to prevent oxidative cell damage might be to take dietary antioxidants that are effective inside of cells. Plant foods contain a large amount of polyphenols whose antioxidative activities have been proved in various chemical assay systems. There have been, however, a limited number of reports indicating that these polyphenols directly suppress oxidative damage in biological assay systems. We tried to find suppressive effects of dietary polyphenols against mammalian and bacterial cytotoxicity induced by H2O2.

Mammalian Assay Systems

Cytotoxic effects of H2O2 and protective effects of polyphenols against H2O2-induced cytotoxicity were assessed by a colony formation assay, one of the most reliable methods of assessing cytotoxic effects (8). In our experiments we avoided direct reactions of polyphenols with H2O2 in the medium in order to evaluate the real effects of the polyphenols in the cell or the cell membrane. Chinese hamster lung fibroblast V79 cells were seeded in 60-mm Petri dishes (200 cells/dish) and incubated in 5 ml of MEM supplemented with 10% heat-inactivated FBS in a humidified atmosphere of 5% CO2 in air at 37°C. After the medium was changed to 5 ml of MEM free of FBS, a polyphenol solution in ethanol (up to 40 μl) was added to the medium, and the cells were incubated for 4 h. After being washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline (pH 7.3), the cells were treated with H2O2 (Its concentration, usually 40-80 μM, depended on the conditions of the cells) in 5 ml of HBS for 30 min. After the cells were cultured in MEM supplemented with 10% FBS for 5 days, the number of colonies was counted. The survival (% of control) was calculated by dividing the number of colonies of the cells treated with polyphenols and/or H2O2 by the number of colonies of untreated control cells. First, we determined the dose dependence of the cytotoxic effects of polyphenols. Then, we adjusted their concentrations so that each polyphenol was itself not toxic.

NDGA, a plant polyphenol derived from Larrea divaricata, is a lipid-soluble antioxidant and an inhibitor of lipoygenases from a wide variety of sources (9) (Fig. 1). Various biological effects of NDGA have been reported. It was found to suppress 12-O-tetradecanoylphorbol-13-acetate-induced chromosomal aberration in human lymphocytes (10) and 12-O-tetradecanoylphorbol-13-acetate-stimulated protein kinase C activity in LLC-PK1 cells (11). Antimutagenic activity in the Ames test and antitumorigenic activity in Sencar mice were also reported (12). We found that NDGA effectively suppressed H2O2 and t-butylhydroperoxide-induced cytotoxicity (7, 13). To the best of our knowledge, this is the first evidence that a plant polyphenol directly inhibits hydroperoxide-induced cytotoxicity. Tetramethoxy-nordihydroguaiaretic acid, an NDGA analogue bearing no phenolic OH (Fig. 1), showed no preventive effect against H2O2-induced cytotoxicity, indicating that phenolic OH is essential for its activity. When the cells were treated with same amount of caffeic acid, ferulic acid, and their methyl esters, only caffeic acid methyl ester prevented the cytotoxicity of H2O2 (14) (Fig. 1). This result indicates that esterification made caffeic acid effective for protection and that substitution of one methoxy for the corresponding phenolic OH of caffeic acid methyl ester diminishes the activity. In other words, o-dihydroxy (catechol) structure seems to be essential for prevention, and it seems difficult for polyphenols bearing free carboxyl groups to be incorporation into the cells or the cell membranes because of electrostatic repulsion owing to the negative charge of the carboxyl group and the membrane phospholipids. The same observation of difference in the suppressive effects applies to gallic acid and its three esters (Fig. 2). The protective activity of methyl, propyl, and lauryl gallate increased in that order (Fig. 3), and gallic acid showed no protective effects, indicating that the affinity of each polyphenol for the cell membrane is critical for protection (14). Since caffeic acid ester and ferulic acid ester have inhibitory effects on lipoygenase at the same degree (15), it is concluded that suppression of H2O2-induced mammalian cytotoxicity cannot be ascribed to lipoygenase inhibition. Flavonoids are another series of polyphenols found in a wide variety of plant sources as vegetables, herbs, nuts, and teas. Various chemical and biochemical effects of flavonoids such as free radical scavengers (16) and inhibitors of lipoygenase, cyclooxygenase, and lipid peroxidation (17, 18) have been reported. But there have been few reports that flavonoids directly suppress H2O2-induced mammalian cytotoxicity.

In our assay system the flavonoids quercetin, kaempferol, catechin, and taxifolin (Fig. 2) suppressed cytotoxicity of H2O2 (19). Quercetin and kaempferol showed protective effects at concentrations 5-50 μM. On the other hand, much higher concentrations of catechin and taxifolin were necessary to prevent the cytotoxicity of H2O2. This difference might be ascribed to their structures depicted in Fig. 2 and affinity for cell membrane. Actually, quercetin is soluble in ethyl acetate. On the other hand, catechin is water soluble. Bors et al. (16) indicated that flavonoids bear three structural groups for radical-
scavenging potential and antioxidative potential: (a) the o-dihydroxy structure in the B ring; (b) the 2,3-double bond in conjugation with the 4-oxo function in the C ring; (c) the 3- and 5-hydroxyl groups with the 4-oxo function in the A and C rings (Fig. 2). Since kaempferol lacks the a group, its preventive effects against the cytotoxicity of active oxygen species can be ascribed to the structural properties of b and c group. On the other hand, the o-dihydroxy moiety in the B ring seems essential for the protective effects of catechin, which lack the b and c groups. Since neither a-tocopherol (Fig. 2) nor ascorbic acid showed any protective effects in our assay systems, radical-scavenging activity is not directly related to the protection. In other words, we can expect new biological activities other than those of the antioxidative vitamins.

**Bacterial Assay Systems**

Mutagenicity and cytotoxicity of H2O2 to *Salmonella typhimurium* TA104, which was developed to detect peroxides and other oxidants (20), were investigated using the preincubation method of the Ames test (21, 22). The bacterial culture (1 ml) and a specific amount of gallic acid ester were incubated at 37°C for 30 min. After being washed by centrifugation, the bacterial solution was incubated with H2O2 at 37°C for 30 min. After being washed by centrifugation again, 0.1 ml of this bacterial solution was mixed with 2.5 ml of top agar and poured on agar plates. Colonies of histidine prototrophs (His+) were counted after incubation at 37°C for 2 days. Viable cells of 10^5-fold diluted bacterial solution were also counted after incubation at 37°C for 2 days. When the culture of *S. typhimurium* TA104 was pretreated with gallic acid esters before challenge with H2O2, the number of surviving colonies increased with the dose of gallic acid esters, indicating that these polyphenols suppress H2O2-induced bacterial cytotoxicity (23). Lauryl gallate was especially effective even at 0.5 μg/plate. In contrast to the esters, gallic acid itself showed no obvious protective effect against the cytotoxicity of H2O2. The effects of gallic acid esters on the numbers of revertants are not as obvious as the effects on survival. If we calculate the RMA by dividing the number of revertants by the number of viable cells, the values of RMA decrease with the dose of gallic acid esters. But the decrease in RMA simply reflects an increase in the number of viable cells rather than a decrease in the number of revertants (23).

Induction of the SOS response by H2O2 was measured in *E. coli* PQ37, whose β-galactosidase expression is strictly dependent on SOS response in spite of constitutive synthesis of alkaline phosphatase (24, 25). After treatment with polyphenols and H2O2, the culture was incubated at 37°C for 2 h, and activities of both β-galactosidase and alkaline phosphatase of each culture were measured. The SOS induction factor is calculated as the ratio of the activities of β-galactosidase and alkaline phosphatase at each measuring point divided by the value of a control culture. The SOS response caused by H2O2-induced DNA lesions in *E. coli* mutants decreased with the addition of gallic acid esters (23). Also in this assay, lauryl gallate was most effective among the three gallic acid esters. In contrast, gallic acid itself showed no effect. Consequently, the protective effects of gallic acid esters against cytotoxicity to *S. typhimurium* TA104 and SOS responses in *E. coli* PQ37 induced by H2O2 supports the idea that the protection of the polyphenols against H2O2-induced cytotoxicity would be common in various assay systems with prokaryote in addition to eukaryote.
Inhibitory Effects against H$_2$O$_2$-induced DNA Strand Breakage in a Model System

The results obtained from the SOS chromotest described above suggests that polyphenols suppress H$_2$O$_2$-induced DNA damage in the cells. It is well known, however, that some polyphenols show genotoxic activities in the presence of transition metal ions such as Fe$^{3+}$ and Cu$^{2+}$ in the medium and that these activities are ascribed to H$_2$O$_2$ formed during autoxidation of the polyphenols (26, 27). It is also reported that caffeic acid causes cellular and isolated DNA damage through H$_2$O$_2$ formation in the presence Cu$^{2+}$ (28, 29). Consequently, these polyphenols have opposite effects on the cytotoxicity of H$_2$O$_2$ in different assay systems. To determine the factors leading to the opposite results, we investigated the effects of states of Fe ions on H$_2$O$_2$-induced DNA single-strand breakage by observing the change of the supercoiled DNA of phage 4r174 into the open circular form in a model system (30). Concerning the amount of transition metals in living cells, such protein-bound forms as heme proteins, ferritin, and transferrin are more common than the “free” forms chelated by low-molecular-weight acids such as citric acid and ADP (31). There are reports that mitochondria are major intracellular targets inactivated by H$_2$O$_2$ (32) and that mitochondrial DNA is modified by prooxidants in the aging process (33, 34). Cytochromes in mitochondria are suggested as possible catalysts in these oxidative damages (35). Thus, we compared the effects of free Fe ion and cytochrome c on H$_2$O$_2$-induced DNA damage. First, we found that H$_2$O$_2$ induced DNA strand break in the presence of cytochrome c. Then, we found that caffeic acid dose dependently inhibited H$_2$O$_2$-induced DNA strand breakage in the presence of cytochrome c. In contrast, caffeic acid caused DNA strand breakage in the presence of free Fe ion even without addition of H$_2$O$_2$. Inhibition by catalase in the latter case indicates that the DNA strand breakage is ascribed to H$_2$O$_2$ formed during autoxidation of caffeic acid. That is, antioxidative and prooxidative activity of caffeic acid toward H$_2$O$_2$-induced DNA strand breakage depends on the state of the Fe ion in the medium. The protective effects of the polyphenols against H$_2$O$_2$-induced cytotoxicity in several assays described in this manuscript suggest, at least, that catalytic roles of free metal ions in autoxidation of polyphenols would not be critical in the cells.

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References

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