Phloroglucinol Attenuates Free Radical-induced Oxidative Stress

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ABSTRACT: The protective role of phloroglucinol against oxidative stress and stress-induced premature senescence (SIPS) was investigated in vitro and in cell culture. Phloroglucinol had strong and concentration-dependent radical scavenging effects against nitric oxide (NO), superoxide anions (O$_2^-$), and hydroxyl radicals. In this study, free radical generators were used to induce oxidative stress in LLC-PK1 renal epithelial cells. Treatment with phloroglucinol attenuated the oxidative stress induced by peroxyl radicals, NO, O$_2^-$, and peroxynitrite. Phloroglucinol also increased cell viability and decreased lipid peroxidation in a concentration-dependent manner. WI-38 human diploid fibroblast cells were used to investigate the protective effect of phloroglucinol against hydrogen peroxide (H$_2$O$_2$)-induced SIPS. Phloroglucinol treatment attenuated H$_2$O$_2$-induced SIPS by increasing cell viability and inhibited lipid peroxidation, suggesting that treatment with phloroglucinol should delay the aging process. The present study supports the promising role of phloroglucinol as an antioxidative agent against free radical-induced oxidative stress and SIPS.

Keywords: phloroglucinol, oxidative stress, nitric oxide, LLC-PK1 cells, WI-38 cells

INTRODUCTION

Oxidative stress and nitrosative stress are caused by the uncontrolled production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (1-3). Excessive production of ROS and RNS can damage cellular lipids, proteins, and DNA, thus inhibiting their normal function. Therefore, oxidative stress and nitrosative stress have been implicated in aging and in a number of human diseases, such as cardiovascular disease, cancer, neurological disorders, and diabetes (4-8). Antioxidants, which prevent free radical damage, have attracted much attention and there has been a marked effort to identify safe and effective therapeutic agents for the treatment of oxidative stress-related diseases. Compelling evidence indicates that increased consumption of dietary antioxidants may improve quality of life by delaying the onset and reducing the risk of degenerative diseases (9-11).

Marine brown algae accumulate a variety of phloroglucinol-based polyphenols in the form of phlorotannins, which consist of phloroglucinol units linked to each other in a variety of ways. Phlorotannins can be found in a variety of marine organisms, especially brown and red algae (12). Several studies have demonstrated that phloroglucinol has broad therapeutic effects, including anti-diabetes (13), antioxidative (14), and anti-cancer (15) effects. Previous work indicates that phloroglucinol significantly decreases the level of radiation-induced intracellular ROS and damage to cellular components such as lipids, DNA, and proteins (16). In addition, phloroglucinol has been reported to reduce ROS levels and increase antioxidant defenses in human HepG2 cells (17). However, the protective effect of phloroglucinol against peroxyl radical- and nitric oxide (NO)-induced oxidative stress in LLC-PK1 cells, which are susceptible to oxidative stress, has not been demonstrated.

Oxidative stress leads to premature senescence in cellular systems, but the effect of phloroglucinol on stress-induced premature senescence (SIPS) in human fibroblast WI-38 cells has not been reported. In the present study, we investigated the protective effect of phloroglucinol against oxidative stress induced by NO and peroxyl radicals. Furthermore, SIPS was induced in human fibroblast WI-38 cells by treatment with hydrogen peroxide (H$_2$O$_2$), and then the protective effect of phloroglucinol against SIPS was investigated.
MATERIALS AND METHODS

Materials
Phloroglucinol, 3-morpholinosydnonimine (SIN-1), pyrogallol, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), 2,2'-Azobisis(2-aminodipropionate) dihydrochloride (AAPH) and sodium nitroprusside (SNP) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The LLC-PK1 porcine renal epithelial cells and WI-38 human embryonic lung-derived diploid fibroblasts were obtained from ATCC (Manassas, VA, USA). Dulbecco’s modified eagle medium/nutrient mixture F-12 (DMEM/F-12), basal medium eagle (BME), and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA).

NO scavenging activity
NO was generated from SNP and measured by the Griess reaction (18) according to the method of Sreejayan and Rao (19). SNP in phosphate buffered saline was mixed with different concentrations of samples and incubated at 25°C for 150 min. The amount of NO produced by SNP was determined by measuring nitrite accumulation with a microplate assay method based on the Griess reaction.

Superoxide anion (O$_2^-$) scavenging activity
O$_2^-$ levels were measured following the method described by Ewing and Janero (20). Briefly, phloroglucinol was added to microplate wells containing 200 μL of freshly prepared 0.125 mM EDTA, 62 μM nitro blue tetrazolium (NBT), and 98 μM NADH in 50 mM phosphate buffer (pH 7.4). The reaction was initiated with the addition of 25 μL of freshly prepared 33 μM 5-methylphenazinium methyl sulfate in 50 mM phosphate buffer (pH 7.4). A microplate reader (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA, USA) was used to monitor NBT reduction by continuously measuring the absorbance (540 nm) of the solution over a 5 min period.

Hydroxyl radical (·OH) scavenging activity
The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM FeSO$_4$-EDTA, 0.15 mL of 10 mM H$_2$O$_2$, 0.525 mL of H$_2$O, and 0.075 mL of sample solution. The reaction was started by the addition of H$_2$O$_2$. After incubation at 37°C for 4 h, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% 2-thiobarbituric acid in 50 mL of 0.05 N NaOH. The solution was boiled for 10 min and then cooled in water. The absorbance of the resulting solution was measured at 520 nm. The ·OH scavenging activity of each sample was defined as the inhibition rate of 2-deoxyribose oxidation by ·OH (21).

Cell culture
Commercially available LLC-PK1 cells were maintained at 37°C in a humidified atmosphere of 5% CO$_2$ in culture plates containing 5% FBS-supplemented DMEM/F-12 medium. The cells were subcultured weekly with 0.05% trypsin-EDTA in calcium- and magnesium-free phosphate buffer. The WI-38 cells were cultured in BME supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37°C and 5% CO$_2$. The cells were subcultured with 0.05% trypsin-EDTA in PBS. Cells at early passages (i.e., between passage 26 and passage 33) were used for all experiments.

Treatment with radical generators
After reaching confluence, LLC-PK1 cells were seeded into 96-well culture plates at a cell density of 1×10$^4$ cells/mL. Two hours later, cells were treated with 1.0 mM AAPH, 1.2 mM pyrogallol, 1.2 mM SNP, or 1.0 mM SIN-1 for 24 h. After the free radical generator treatments, cells were treated with various concentrations of phloroglucinol for 24 h (22-23). The WI-38 cells were seeded into 96-well plates at a cell density of 1×10$^4$ cells/mL and then treated with 50 μM of H$_2$O$_2$ for 60 min to induce SIPS. After SIPS induction, WI-38 cells were treated with various concentrations of phloroglucinol for 24 h.

MTT assay
Cell viability was determined with a MTT colorimetric assay (24). Fifty microliters of MTT solution (1 mg/mL) were added to the each well. After incubation for 4 h at 37°C, the MTT solution was removed from the cells. The formazan crystals present in the renal cells were solubilized with 100 μL of dimethylsulfoxide. A microplate reader (SpectraMax 340PC) was used to read the absorbance of each well at 540 nm.

Thiobarbituric acid reactive substances (TBARS)
The level of lipid peroxidant released from cultured cells was estimated as TBARS according to the methods of Yagi (25) and Yokode et al. (26) with a slight modification. One aliquot of medium was mixed with 1.5 mL of 0.67% thiobarbituric acid aqueous solution and 1.5 mL of 20% trichloroacetic acid. The resulting mixture was boiled at 95 ∼ 100°C for 45 min and then cooled and shaken vigorously with 3.0 mL of n-butanol. After centrifuging at 4,000 g for 10 min, a fluorescence spectrophotometer was used to measure the fluorescence of the n-butanol layer (excitation wavelength: 515 nm, emission wavelength: 553 nm).
Statistical analysis
The results for each group are expressed as mean±standard deviation. SAS software (SAS Institute Inc., Cary, NC, USA) was used to analyze the differences between the control and sample treated groups. Differences between groups were considered significant when the P-value was less than 0.05.

RESULTS

In vitro radical scavenging activity
Table 1 shows the in vitro radical scavenging activities of phloroglucinol. Phloroglucinol scavenged NO, O$_2^-$, and ·OH radicals in a dose-dependent manner. The radical scavenging effect of phloroglucinol was strongest against ·OH radicals in a dose-dependent manner. The radical scavenging effects against O$_2^-$ concentration was associated with 55.5% and 59.1% scavenging effects against O$_2^-$ and ·OH, respectively. In addition, the 1,000 μg/mL phloroglucinol concentration was associated with 55.5% and 59.1% scavenging effects against O$_2^-$ and ·OH, respectively.

Protective activity against radical-induced oxidative stress
The effect of phloroglucinol on the viability and lipid peroxidation of LLC-PK1 renal tubular epithelial cells treated with AAPH is shown in Table 2. Treatment with 1 mM AAPH for 24 h reduced the viability of LLC-PK1 cells to 16.40%. However, phloroglucinol treatment exerted a protective effect against AAPH-induced cellular damage. Treatment with 25 μg/mL, 50 μg/mL, and 100 μg/mL phloroglucinol elevated cell viability from 16.40% to 59.75%, 71.70%, and 95.6%, respectively. Treatment with AAPH increased lipid peroxidation in LLC-PK1 renal tubular epithelial cells, resulting in a TBARS concentration of 0.80 nmol/mg protein. However, treatment with phloroglucinol significantly decreased the formation of TBARS in a concentration-dependent manner. The addition of 25 μg/mL and 100 μg/mL phloroglucinol to AAPH treated wells decreased lipid peroxidation, leading to TBARS concentrations of 0.43 nmol/mg protein and 0.21 nmol/mg protein, respectively.

Pyrogallol was used to evaluate the protective activity of phloroglucinol against O$_2^-$ and ·OH. As shown in Table 3, O$_2^-$ generated by pyrogallol decreased cell viability from 100% to 31.90%, and elevated malondialdehyde (MDA) formation from 0.21 nmol/mg protein to 0.76 nmol/mg protein. However, treatment with a 100 μg/mL phloroglucinol concentration increased cell viability to 79.1% and decreased MDA levels to 0.23 nmol/mg protein.

Table 4 shows the protective effect of phloroglucinol.
against NO-induced oxidative stress. The viability of cells that had been treated with SNP was markedly lower than that of non-treated cells. However, phloroglucinol increased cell viability in a dose-dependent manner. At a 100 μg/mL phloroglucinol concentration, cell viability was elevated to 80.1%. In addition, SNP treatment increased lipid peroxidation from 0.22 nmol MDA/mg protein to 0.80 nmol MDA/mg protein, while treatment with 100 μg/mL phloroglucinol decreased lipid peroxidation to 0.32 nmol MDA/mg protein.

Table 5. Protective effect of phloroglucinol against SIN-1-induced oxidative stress in LLC-PK1 cells

| Concentration (μg/mL) | Cell viability (%) | MDA (nmol/mg protein) |
|-----------------------|-------------------|----------------------|
| SIN-1 non-treated control | 100.00±0.66a       | 0.19±0.01c           |
| SIN-1-treated control   | 21.40±1.32c        | 0.81±0.01c           |
| 10                    | 52.80±1.32e        | 0.70±0.01c           |
| 25                    | 66.00±1.32d        | 0.56±0.01c           |
| 50                    | 71.28±1.98c        | 0.34±0.02d           |
| 100                   | 85.80±1.98b        | 0.20±0.01c           |

Values are mean±SD.

“Means with different superscripts within each column are significantly different (P<0.05) by Duncan’s multiple range test. Two hours after seeding, LLC-PK1 cells were treated with 1.0 mM SIN-1 for 24 h and then 10, 25, 50, or 100 μg/mL phloroglucinol for 24 h.

Table 6. Protective effect of phloroglucinol against H2O2-induced SIPS in WI-38 fibroblast cells

| Concentration (μM) | Cell viability (%) | MDA (nmol/mg protein) |
|--------------------|--------------------|----------------------|
| Young control      | 100.00±2.33a       | 0.45±0.02c           |
| Premature senescence control | 64.60±1.45c | 1.02±0.01c           |
| 25                 | 79.98±1.54a        | 0.57±0.01c           |
| 50                 | 85.36±1.77c        | 0.48±0.01d           |
| 100                | 91.65±1.56b        | 0.42±0.02c           |

Values are mean±SD.

“Means with different superscripts within each column are significantly different (P<0.05) by Duncan’s multiple range test. Two hours after seeding, WI-38 cells were treated with 50 μM H2O2 for 60 min and then treated with 10, 25, 50, or 100 μg/mL phloroglucinol for 24 h.

and 100 μg/mL concentrations of phloroglucinol were 0.57 nmol/mg protein and 0.42 nmol/mg, respectively.

**DISCUSSION**

Phloroglucinol is the monomeric building unit of phlorotannins, which are phenolic compounds that can be found in brown algae. The free form of phloroglucinol can be found in some members of the order Fucales, including Cystoseira discors (>0.5% dry weight (DW)) (27), Cystoseira tamariscifolia (>0.5% DW) (27), and Ecklonia cava (Laminariaceae) (0.016% DW) (28).

Several studies have reported on the biological activities of phloroglucinol, which can be isolated from brown algae or purchased from Sigma Chemical Co. Kang et al. (28) demonstrated that phloroglucinol from Ecklonia cava has a ROS scavenging effect and protects Chinese hamster lung fibroblast (V79-4) cells against oxidative damage by enhancing cellular catalase activity and regulating extracellular signal-regulated kinase. In addition, Ishii et al. (29) demonstrated that phloroglucinol and phloroglucinol derivatives from Mallotus japonicus inhibit NO production by reducing inducible NO synthase protein induction and inhibiting enzyme activity in lipopolysaccharide- and interferon-γ-stimulated RAW 264.7 cells. Quéguiner et al. (17) reported that phloroglucinol from Sigma Chemical Co. has antioxidant activities in non-biological assays and alleviates oxidative stress in human HepG2 cells. Phloroglucinol has a direct scavenging effect against free radicals, increases antioxidant enzyme defenses, and protects against lipid peroxidation in cultivated liver cells. However, the protective role of phloroglucinol against free radical overproduction by LLC-PK1 renal epithelial cells, which are susceptible to oxidative stress, has not been studied. In addition, the effect of phloroglucinol on SIPS in human fibroblast WI-38 cells has not been reported. In the present study, we investigated the protective effects of phloroglucinol...
against oxidative stress and SIPS in vitro and in cell culture.

Since the overproduction of free radicals results in a variety of pathological conditions, including cardiovascular disease, cancer, neurological disorders, diabetes, and aging. Antioxidants that prevent free radical damage have attracted much attention (30,31). Several studies have demonstrated that brown seaweeds play a protective role against pathological phenomena. Because phloroglucinol is one of the main active components of brown seaweeds (12-15), the present investigation was focused on the protective activity of phloroglucinol against free radical-induced oxidative stress in vitro and in cell culture.

NO is a free radical with a single unpaired electron. Overproduction of NO causes tissue damage and contributes to pathological conditions (30,31). In addition, reaction of O$_2^-$ with NO leads to the formation of ONOO$^-$, which is far more reactive and toxic than its precursors (32,33). This reaction is extremely rapid, and can generate the most toxic radical, ·OH (34). ·OH is the most reactive radical, and it can react with the unsaturated fatty acids of membrane phospholipids to generate free radicals, which in turn react quickly with oxygen to form peroxides. These peroxides then act as free radicals, initiating an autocatalytic chain reaction that results in further loss of unsaturated fatty acids and extensive membrane damage. In this study, we found that phloroglucinol had strong and concentration-dependent radical scavenging effects against NO, O$_2^-$ and ·OH. The present results provide evidence to indicate that phloroglucinol plays a protective role against oxidative stress by scavenging free radicals. The use of LLC-PK1 cells, which are vulnerable to the oxidative stress induced by free radicals, as a cellular model of oxidative stress is well established and is useful when searching for agents that can provide effective protection from free radicals (22,23). Therefore, in the present study, cellular oxidative stress was induced in LLC-PK1 cells by free radical generators such as AAPH, SNP, pyrogallol, and SIN-1.

AAPH-intoxication experiments are useful for evaluating the biological activities of antioxidants. AAPH generates free radicals at a constant and well-defined rate by its thermal decomposition without biotransformation (35). The free radicals generated from AAPH rapidly react with oxygen molecules to yield peroxyl radicals. The lipid peroxy radicals attack other lipid molecules to form lipid hydroperoxides and new lipid radicals. This reaction takes place repeatedly, with resultant attacks upon various biological molecules, and induces physico-chemical alterations and cellular damage (36). In the present study, we used AAPH to produce peroxy radicals. The present study showed that AAPH leads to a decline in cell viability and an elevation in lipid peroxidation in LLC-PK1 renal epithelial cells. However, treatment with phloroglucinol exerted a protective effect against AAPH-induced oxidative damage to LLC-PK1 cells, resulting in a dose-dependent increase in cell viability and decrease in lipid peroxidation.

The protective activity of phloroglucinol against NO-induced oxidative stress was also evaluated. NO and O$_2^-$ generated by SNP and pyrogallol, respectively, significantly decreased cell viability and elevated lipid peroxidation compared to non-treated cells. However, phloroglucinol had significant protective effects against NO- and O$_2^-$-induced cell loss and lipid peroxidation. Peroxynitrite, which is derived from the reaction of NO with O$_2^-$, is a strong oxidant and nitrating agent that can lead to the production of the highly toxic ·OH radical (32). SIN-1 simultaneously generates both NO and O$_2^-$, which then rapidly combine to form ONOO$^-$ (23). Compared with non-treated cells, treatment with SIN-1 led to a significant decrease in cell viability and increase in lipid peroxidation. However, phloroglucinol attenuated this oxidative stress by increasing cell viability and decreasing lipid peroxidation. These results indicate that phloroglucinol plays a protective role against NO-induced oxidative stress.

Oxidative reactions that occur in organisms generate free radicals, which cause multiple lesions in macromolecules and lead to aging (37,38). In addition, a decline in antioxidant defenses and progressive increase in oxidative stress is commonly associated with aging and age-related degenerative diseases. Human diploid fibroblasts (HDFs) are a typical model for studying the process of aging in vitro. Various oxidative stresses, such as treatment with H$_2$O$_2$, have been used to study the onset of cellular senescence (39,40). Exposure of normal HDFs to H$_2$O$_2$ is widely used as a model to study cellular aging. The exposure of HDFs to a sublethal concentration of H$_2$O$_2$ induces permanent cell cycle arrest and phenotypic changes that mimic those seen in replicatively senescent cells (41). Therefore, an aging model of H$_2$O$_2$-induced SIPS was used to investigate the protective effect of phloroglucinol against the oxidative stress induced by H$_2$O$_2$. In the present study, H$_2$O$_2$-induced SIPS led to a loss of cell viability, suggesting that H$_2$O$_2$ treatment led to premature replicative senescence (41,42). Treatment with phloroglucinol improved cell viability by protecting against H$_2$O$_2$-induced oxidative damage. Intracellular oxidative stress has harmful effects on biomolecules such as lipids, proteins, and DNA and eventually leads to cellular senescence.

Previously, Morliere and Santus (43) reported that the exposure of cultured human skin fibroblasts to tert-buthyl hydroperoxide, ultraviolet-A, and H$_2$O$_2$ results in the release of high levels of TBARS, an index of lipid pero-
xidation. MDA is used as a biological marker of lipid peroxidation. In the present study, MDA levels were significantly increased by exposure to H_2O_2. In contrast, treatment with phloroglucinol inhibited lipid peroxidation in WI-38 cells under SIPS, suggesting that phloroglucinol may protect against oxidative stress. Although further study into the mechanism responsible for this protective effect has to be carried out, the present study supports the promising role of phloroglucinol as an anti-oxidative agent against free radical-induced oxidative stress and SIPS.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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