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Effects of Resveratrol and 4-hexylresorcinol on Hydrogen Peroxide-induced Oxidative DNA Damage in Human Lymphocytes

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The protective effects of resveratrol and 4-hexylresorcinol against oxidative DNA damage in human lymphocytes induced by hydrogen peroxide were investigated. Resveratrol and 4-hexylresorcinol showed no cytotoxicity to human lymphocytes at the tested concentration (10–100 μM). In addition, DNA damage in human lymphocytes induced by H_2O_2 was inhibited by resveratrol and 4-hexylresorcinol. Resveratrol and 4-hexylresorcinol at concentrations of 10–100 μM induced an increase in glutathione (GSH) levels in a concentration-dependent manner. Moreover, these two compounds also induced activity of glutathione peroxidase (GPX) and glutathione reductase (GR). The activity of glutathione-S-transferase (GST) in human lymphocytes was induced by resveratrol. Resveratrol and 4-hexylresorcinol inhibited the activity of catalase (CAT). These data indicate that the inhibition of resveratrol and 4-hexylresorcinol on oxidative DNA damage in human lymphocytes induced by H_2O_2 might be attributed to increase levels of GSH and modulation of antioxidant enzymes (GPX, GR and GST).

Keywords: Resveratrol; 4-Hexylresorcinol; Antioxidant enzyme; DNA damage; Lymphocyte

INTRODUCTION

Oxidative stress exerted by all peroxides can directly damage cells and tissues.[1] To defend against oxidative stress caused by oxygen toxicity, many endogenous non-protein small antioxidant molecules and antioxidant enzymes are considered essential for alleviating oxidative stress by acting as chain-breakers of the oxygen radical cascade and lipid peroxidation chain reaction. The primary antioxidant enzymes that minimize the oxygen radical cascade and remove cytotoxic peroxides are those such as glutathione peroxidase (GPX), glutathione reductase (GR), glutathione transferase (GST), catalase (CAT), superoxide dismutase (SOD), etc.[2] These antioxidant enzymes prevent the formation of O_2 radicals and alleviate the lipid peroxidation. In addition, glutathione (GSH) is widely distributed in plants, animals and microorganisms. The detoxicant, antioxidant, and cysteine-reservoir functions of cellular GSH, which are the potential of this ubiquitious thiol to modulate cellular signal transduction processes have been recently demonstrated. In addition, GSH is well known to have multifaceted physiological functions.

Active oxygen species are produced during normal cell metabolism as well as after exposure to various physical and chemical agents. However, the primary antioxidant enzymes (SOD, GPX, GR, GST) and GSH prevent cells from attack by active oxygen species.[3] Some literature[4] has reported that antioxidant micronutrients such as vitamin C, vitamin E and β-carotene in fruits and vegetables contributes in part to anticarcinogenic protection. In recently years, scientific attention has investigates

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the significance of other minor dietary compounds.[5] Thus, prevention of disease and ageing by the diet containing biofunctional molecular has become more and more important.

Resveratrol (3,4′,5-trihydroxystibene), a phytoalexin found in grapes and other food products, acts as an antioxidant and antimutagen and to induce phase II drug-metabolizing enzymes. It mediated anti-inflammatory effects and inhibited cyclooxygenase and hydroperoxidase functions. In addition, resveratrol was found to induce quinone reductase activity and to inhibit cellular events associated with tumor initiation, promotion and progression.[6] 4-Hexylresorcinol (4-hexyl-1,3-benzenediol), widely used in several Mediterannean countries, has been used as an anthelmintic and antiseptic in human medicine.[7] It was also used as the inhibitor of the polyphenol oxidase and to inhibit melanosis (black sports) in shrimp.[8] These studies suggested that resveratrol and 4-hexylresorcinol might act as potential cancer chemopreventive agents in humans. Although these two compounds have shown many physiological properties, still very little is known about their biological effects on modulating the GSH and antioxidant enzymes in normal cells. Thus, the objective of this study was to investigate the effects of these two compounds in protecting against oxidative DNA damage in human lymphocytes and in modulation of antioxidant enzymes were also evaluated.

MATERIALS AND METHODS

Chemicals

Resveratrol, butylated hydroxyanisole (BHA), *N*-laurory sarcosinate, ethidium bromide, ferrozine and trypan blue, Titron X-100 and trypan blue were supplied by Sigma Chemical Co. (St. Louis, MO). Agarose, Histopaque 1077 separation media, RPMI-1640, Dubeccos' Modeified Eagle Medium, fetus bovine serum (FBS), trypsin-EDTA(T/E), penicillin-streptomycin, L-glutamine, MEM sodium pyruvate solution, Ultrapure low melting point agarose (LMA), normal melting point agarose (NMA) and MEM non-essential amino acid (NEAA) were supplied by Gibco Life Co. Ltd. (Grand Island, NY). Bloods were supplied from healthy volunteers.

Blood Samples

Blood samples were obtained from healthy volunteers. Blood was drawn by venipuncture and heparinized with a calparine. Red blood cells and granulocytes were removed from undiluted whole blood using a cell separation tube. Lymphocytes were separated using a separation medium with a density of 1.007 g/ml, composed of an aqueous solution of Ficoll (57 g/l). The purity of prepared lymphocytes was greater than 96% as determined by Giemsa staining. In addition, lymphocytes cytosol, supernatant of the cells preincubated with 10–100 μM of resveratrol or 4-hexylresorcinol at 37°C for 30 min, was prepared for antioxidant enzyme assay.

Cytotoxicity Analysis

For the cytotoxicity studies, cells were treated with different concentrations (10–100 μM) of resveratrol or 4-hexylresorcinol. Cells were incubated at 37°C for 30 min. After incubation, cells were stained with 5 μl trypan blue. Cell number and viability were determined by using a Neubauer improved haemocytometer before and after samples were treated.

Lipid Peroxidation in Human Lymphocytes

The determination of lipid hydroperoxide in human lymphocytes was measured according to Chirico *et al.*[9] with slight modification. Lymphocytes were incubated with 10–100 μM of resveratrol or 4-hexylresorcinol at 37°C for 30 min. After incubation, BHA was added to the reaction mixture. One milliliter of 2.8% trichloroacetic acid (TCA) and 1 ml of 1% thiobarbituric acid (TBA) were added to the mixture, which was them heated in a water bath at 100°C for 2 min. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm. The control sample was without resveratrol or 4-hexylresorcinol.

Single-cell Gel Electrophoresis (Comet Assay)

The comet assay was performed under alkaline conditions following to the methods of Duthie *et al.*[5] Lymphocytes were treated with various concentrations of resveratrol or 4-hexylresorcinol (10–100 μM) in combination with 50 μM hydrogen peroxide for 30 min at 37°C in an incubator together with untreated control samples. Negative control received the equivalent concentration of DMSO only. Lymphocytes were treated with hydrogen peroxide at the same incubation condition as the positive control. After incubation, the lymphocytes were harvested by centrifugation at 800–900 rpm at 4°C for 5 min. The cells were embedded in agarose, lysed and electrophoresed, then the slides were stained with ethidium bromide (EtBr) and observed using a fluorescent microscope attached to a CCD camera connected to a personal computer based image analysis system (Komet 3.0; Kinetic Imaging Ltd.). One hundred cells on each slide (scored at random) were classified according to the relative intensity of fluorescence in the tail. The degree of DNA damage

was scored by tail moment (TM; tail moment = tail length × tail DNA%/100).

Determination of Reduced Glutathione (GSH)

The determination of GSH levels in lymphocytes was carried out according to the method of Hu *et al.*[10] The lymphocytes were preincubated with 10–100 μM of resveratrol or 4-hexylresorcinol at 37°C for 30 min. After incubation, 0.2 ml of reaction mixture was added 0.8 ml 5% TCA in ice for 5 min. The 0.3 ml of supernatant was mixed with 0.7 ml Tris/EDTA (pH 8.9), then 20 ml 5.5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) was added and reacted for 5 min. The GSH levels were determined spectrophotometrically at 412 nm.

Glutathione Peroxidase (GPX) Assay

GPX activity was measured as described by Lawrence and Burk.[11] H_2O_2 was used as the substrates for GPX determination. The assay mixture consisted of 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 U/ml GSH reductase, 1 mM potassium phosphate buffer (pH 7.0), 0.1 ml 2.5 mM H_2O_2 and lymphocytes cytosol. Enzyme activity was calculated by the change of the absorbance value at 340 nm for 5 min. GPX activity was expressed as nmol/NADPH/mg protein.

Glutathione *S*-transferase (GST) Assay

GST activity was determined spectrophotometrically according to Habig *et al.*[12] GST activity was measured using 0.1 ml of lymphocytes cytosol, 1 mM 1-chloro-2,4-dinitrobenzene, and 1 mM GSH as substrate in a final volume of 1 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 37°C. Enzyme activity was calculated by the change of the absorbance value at 340 nm for 5 min. GST activity was expressed as nmol/min/mg protein.

Glutathione Reductase (GR) Assay

GR activity was determined according to Bellomo *et al.*[13] The reaction mixture consisted of 1.1 mM magnesium chloride, 100 mM phosphate buffer (pH 7.0), 0.1 mM NADPH, 5 mM GSSG and lymphocytes cytosol. Oxidation of NADPH was monitored spectrophotometrically. GR activity was calculated by the change of the absorbance value at 340 nm for 5 min, GR activity was expressed as nmol/min/mg protein.

Catalase (CAT) Assay

CAT activity was measured according to the method of Cohen *et al.*[14] The reaction mixture consisted of 0.1 ml lymphocytes cytosol, 0.9 ml 50 mM potassium phosphate butter (pH 7.0), and 50 mM H_2O_2. For the control, the phosphate buffer was added instead of H_2O_2. The decomposition of H_2O_2 was recorded as 240 nm for 2 min. CAT activity was calculated using the rate constant.

Statistical Analysis

All analyses were run in triplicate and averaged. Statistical analyses were performed according to the SAS Institute User's Guide. Analyses of variance were performed using the ANOVA procedure. Significant differences ($P < 0.05$) between the means were determined using Duncan's multiple range test.

RESULTS AND DISCUSSION

The cytotoxicity of resveratrol and 4-hexylresorcinol to human lymphocytes was evaluated. The cell viability was greater than 95% when resveratrol and 4-hexylresorcinol (10–100 μM) were incubated with cells at 37°C for 30 min (data not shown). This implies that resveratrol and 4-hexylresorcinol showed no cytotoxicity to human lymphocytes at the tested concentrations.

The effects of resveratrol and 4-hexylresorcinol on lipid hydroperoxide induced with or without hydrogen peroxide in human lymphocytes were evaluated. No significant difference ($P > 0.05$) in the production of lipid hydroperoxides was found in human lymphocytes induced with or without hydrogen peroxide (50 μM). Resveratrol and 4-hexylresorcinol at the concentration 0–100 μM tested did not promote the formation of lipid hydroperoxides (data not shown). Therefore, resveratrol and 4-hexylresorcinol not only showed no cytotoxcity to human lymphocytes but also showed no promotion of lipid peroxidation. However, the effects of resveratrol and 4-hexylresorcinol on DNA in human lymphocytes remain unclear.

Figure 1 shows the effects of resveratrol and 4-hexylresorcinol on hydrogen peroxide-inuced DNA damage in human lymphocytes. The results show that these two compounds do not cause DNA damage at concentrations of 10–100 μM as compared with the control group. At a concentration of 25 μM, the cytoprotective ability of resveratrol and 4-hexylresorcinols were 19 and 9%, respectively. As for 4-hexylresorcinol (10–100 μM), less then 10% inhibitory effect on DNA damage induced by H_2O_2 treatment was found. Clearly, resveratrol showed more protection against DNA damage in human lymphocytes induced by H_2O_2 than did 4-hexylresorcinol.

Hydrogen peroxide is not a radical, since it has no unpaired electrons, and it displays a moderate chemical reactivity. Hydrogen peroxide, a potent apoptosis-inducing agent, can freely cross biological membranes and it is believed to cause DNA strand breakage by generation of the hydroxyl radical close to DNA molecule, via the Fenton reaction.[5,15] The scavenging effect of resveratrol and 4-hexylresorcinol on hydrogen peroxide was also investigated (data not shown). Resveratrol and 4-hexylresorcinol at concentrations of 10 μM have no apparent scavenging effect on hydrogen peroxide, whereas resveratrol and 4-hexylresorcinol at 100 μM showed 2 and 5% scavenging effect, respectively. In other words, hydrogen peroxide was not removed by resveratrol and 4-hexylresorcinols. Consequently, the protective mechanism against DNA damage induced by H_2O_2 may be associated with modulation of antioxidant enzyme activities.

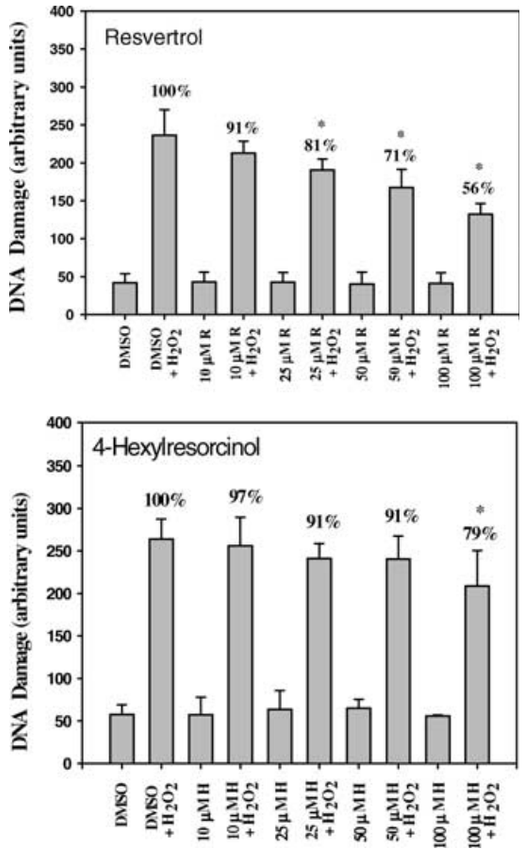

FIGURE 1 Effects of resveratrol and 4-hexylresorcinol on hydrogen peroxide-induced DNA damage in isolated human lymphocytes. Human lymphocytes were incubated for 30 min with 0–100 μM resveratrol (R) or 4-hexylresorcinol (H) and 50 μM hydrogen peroxide (H_2O_2). DNA strand breaks were measured using the comet assay. The inhibition of DNA damage by resveratrol and 4-hexylresorcinol is shown as a percentage. Results are mean ± SEM for $n \geq 3$. *$P \leq 0.05$ refers to differences between hydrogen peroxide-treated lymphocytes incubated with or without resveratrol and 4-hexylresorcinol.

The effects of resveratrol and 4-hexylresorcinols on GSH levels in human lymphocytes are shown in Table I. Resveratrol and 4-hexylresorcinol at concentrations of 10–100 μM induced an increase in GSH level in a concentration-dependent manner as compared with the control group. The increase in GSH levels in a concentration-dependent manner by resveratrol was more than that by 4-hexylresorcinols. GSH is known to have multifaceted physiogical functions, and the side chain sulfhydryl (–SH) residue in cysteine of GSH accounts for most of its physiological properties.[16] Some studies have reported that vitamin C supplementation increased men red blood cell GSH content by about 50%.[16,17] In addition, ascorbate may decrease GSH consumption by minimizing the GSH dependent reduction of dehydroascorbate to ascorbate, and by providing an alternative cellular reducing agent.[18] In the present study, increases in the GSH levels may be due to the reducing property of resveratrol and 4-hexylresorcinol.

Glutathione peroxidase (GPX) has long been regarded as a crucial enzyme for the removal of cytotoxic hydroperoxides, and it has an absolute requirement for the hydrogen donor GSH for catalytic activity. This enzyme (GPX) may use a wide range of substrates extending from H_2O_2 to organic hydroperoxides. Hence, GPX is one of the primary antioxidant enzymes which are considered essential for alleviating oxidative stress by acting as chain-breakers of the O_2 radical cascade and lipid peroxidation chain reaction.[19] Table II shows the effects of resveratrol and 4-hexylresorcinols on GPX activity in human lymphocytes. Resveratrol and 4-hexylresorcinol at a concentration of 10–100 μM were found to significantly induce GPX activity in human lymphocytes as compared with the control group. These two compounds induced GPX activity in a concentration-dependent manner in human lymphocytes. Resveratrol and 4-hexylresorcinol at a concentration of 100 μM induced 38 and 44% GPX activity, respectively. Clearly, these two compounds are highly effective inducers of GPX activity.

TABLE I Effects of resveratrol and 4-hexylresorcinol on glutathione content in human lymphocytes

	Glutathione (nmol/mg protein)*	
Concentration (μM)	Resveratrol	4-Hexylresorcinol
Control	11.3 ± 0.5	11.0 ± 0.4
10	13.1 ± 0.6**	12.2 ± 0.4**
25	14.2 ± 0.7**	12.8 ± 0.2**
50	15.4 ± 0.2**	13.4 ± 0.2**
100	16.9 ± 0.5**	15.3 ± 0.3**

*Values are means ± SEM of triplicate determinations of DTNB conjugated formed. Cell homogenates from freshly isolated human lymphocytes were preincubated with 0–100 μM resveratrol or 4-hexylresorcinol for 30 min, respectively. **Significantly different from the control ($P < 0.05$).

TABLE II Effects of resveratrol and 4-hexylresorcinol on GPX activity in human lymphocytes

	Activity (μmol/min/mg protein)*	
Concentration (μM)	Resveratrol	4-Hexylresorcinol
Control	124.8 ± 3.3	123.4 ± 4.5
10	124.5 ± 4.4	140.5 ± 6.6**
25	149.3 ± 2.4**	158.8 ± 5.4**
50	154.9 ± 2.2**	167.2 ± 4.5**
100	172.6 ± 4.4**	177.5 ± 2.6**

*Values are means ± SEM of triplicate determinations by following the oxidation of NADPH. Cell homogenates from freshly isolated human lymphocytes were preincubated with 0–100 μM resveratrol or 4-hexylresorcinol, respectively, for 30 min. **Significantly different from the control ($P < 0.05$).

This finding is significant because these two compounds are able to alleviating oxidative stress and lipid peroxidation in cells.

The effects of resveratrol and 4-hexylresorcinol on GR activity in human lymphocytes are shown in Table III. These two compounds were not able to induce GR activity in human lymphocytes at concentrations less than 10 μM, and they began to effectively induce the GR activity in human lymphocytes at greater then 10 μM. The GR activities in human lymphocytes exposed to resveratrol and 4-hexylresorcinols was increased 31 and 15%, respectively, at a concentration of 100 μM. GR is a crucial enzyme for the regeneration of GSH from GSSG, so GSH levels are associated with GR activity. The more activity of GR there is in cells, the greater is the GSH level in cells. In the present study, resveratrol and 4-hexylresorcinol increased GR activity; consequently, GSH is significantly generated intracellularly from its oxidized form, glutathione disulfide (GSSH), by GR in the presence of NADPH. This result may account for an increase in the GSH levels in human lymphocytes.

Resveratrol and 4-hexylresorcinol at concentrations of 10–100 μM, except 100 μM resveratrol, were unable to induce GST activity in human lymphocytes (data not shown). GST activity in human lymphocytes exposed to 100 μM resveratrol showed an significant increase of 17%, although GST activity was not affected by 4-hexylresorcinol at 100 μM. The detoxification mechanism of GST promotes binding of the –SH group of GSH with electrophilic compounds. This makes the conjugated compound more water-soluble and more easily excreted from the cells.[20] Wu *et al.*[21] reported that water extracts of unroasted and 150°C -roasted *Cassia tora* L. at 1 mg/ml promoted enzyme activation in HepG2 cells. Hu and Singh[10] showed that sulfide, diallyl trisulfide, and dipropyl sulfide in garlic enhanced the GST activity of A/J rats and reduced the rate of lung cancer of rats under high dosage of B(a)P. The anticancer properties of extract of rosemary were related to its promotion of GST activities.[22] Resveratrol was found to act as an antioxidant, antimutagen, and to induce phase II drug-metabolizing enzymes; in addition, it mediated anti-inflammatory effects and induced antipromotion activities. These properties may contribute to its cancer chemopreventive activity.[6] In the present study, the GST activity induced by resveratrol at a higher concentration (100 μM) might be another mechanism against genotoxicity in cells.

Table IV shows the effects of resveratrol and 4-hexylresorcinol on CAT activity in human lymphocytes. CAT activity treated with 10–100 μM of resveratrol and 4-hexylresorcinol was inhibited, as compared with the control group. CAT activity might be inhibited because resveratrol and 4-hexylresorcinols complex with proteins through hydrogen and covalent bonds, causing precipitation and/or enzyme inhibition.[23,24] Hydrogen peroxide is detoxified by peroxidase and/or CAT as a result of catalyzing the removal of the H_2O_2. Hence, CAT may play an important role in protecting against dietary oxidative stress in insects and other animals. In the present study, it can be clearly seen that the CAT activity was inhibited by these two compounds. Thus, the ability of resveratrol and 4-hexylresorcinol to inhibit CAT might have greater toxicological implication by inhibiting a key antioxidant enzyme and producing toxic oxygen species, and thereby

TABLE III Effects of resveratrol and 4-hexylresorcinol on glutathione reductase activity in human lymphocytes

	Activity (μmol/min/mg protein)*	
Concentration (μM)	Resveratrol	4-Hexylresorcinol
Control	30.0 ± 0.7	30.6 ± 0.6
10	30.1 ± 0.6	30.7 ± 0.8
25	32.2 ± 0.6**	32.6 ± 0.4**
50	34.4 ± 0.7**	33.8 ± 0.3**
100	39.4 ± 0.5**	35.3 ± 0.7**

*Values are means ± SEM of triplicate determinations by following the oxidation of NADPH. Cell homogenates from freshly isolated human lymphocytes were preincubated with 0–100 μM resveratrol or 4-hexylresorcinol, respectively, for 30 min. **Significantly different from the control ($P < 0.05$).

TABLE IV Effects of resveratrol and 4-hexylresorcinol on CAT activity in human lymphocytes

	Activity (μmol/min/mg protein)*	
Concentration (μM)	Resveratrol	4-Hexylresorcinol
Control	50.2 ± 0.5	50.8 ± 0.8
10	50.0 ± 0.6	45.2 ± 0.7**
25	49.2 ± 0.4	44.6 ± 0.4**
50	44.2 ± 0.8**	39.6 ± 0.7**
100	40.9 ± 0.7**	32.6 ± 1.0**

*Values are means ± SEM of triplicate determinations by following the decomposition of hydrogen peroxide. Cell homogenastes from freshly isolated human lymphocytes were preincubated with 0–100 μM resveratrol or 4-hexylresorcinol, respectively, for 30 min. **Significantly different from the control ($P < 0.05$).

promoting extensive oxidative stress *in vivo*.[23] In contrast, GPX, GR and GST activities were induced by resveratrol and 4-hexylresorcinol. The explanation for different reactions between CAT and other enzymes with resveratrol and 4-hexylresorcinols merits further investigation.

In conclusion, the inhibition of resveratrol and 4-hexylresorcinol on oxidative damage in human lymphocytes induced by hydrogen peroxide could be due to the increase the GSH levels and their abilities to modulate the antioxidant enzymes (GPX, GR and GST) except CAT. In addition, the antioxidant enzymes activity induced by resveratrol and 4-hexylresorcinol should protect cells from oxidative damage. The biological implications of these finding could be important not only for understanding antioxidant properties, but also in understanding the modulating antioxidant enzymes of resveratrol and 4-hexylresorcinol.

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