Antioxidant Effect of Homogentisic Acid on Hydrogen Peroxide Induced Oxidative Stress in Human Lung Fibroblast Cells

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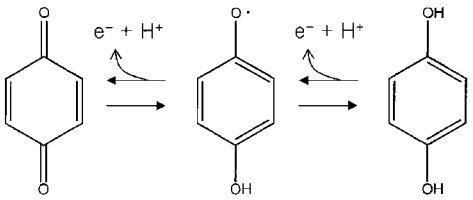
Abstract Homogentisic acid was found to scavenge intracellular reactive oxygen species (ROS), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and thus prevented lipid peroxidation in human fibroblast (WI 38) cells. The radical scavenging activity of homogentisic acid was found to protect WI 38 cells against hydrogen peroxide (H_2O_2) induced oxidative stress, via the activation of extracellular signal regulated kinase (ERK) protein. Homogentisic acid increased the activity of catalase. Hence, from the present study, it is suggested that homogentisic acid protects WI 38 cells against H_2O_2 damage by enhancing the intracellular antioxidative activity.

Keywords: homogentisic acid, oxidative stress, catalase

INTRODUCTION

An antioxidant can be broadly defined as "any substance, which at concentrations lower than oxidizable substrate, significantly delays or prevents oxidation" [1]. 1,4-Dihydroxybenzene derivatives (hydroquinones) are easily oxidized to 1,4-benzoquinones (quinones) by the action of oxidants. Quinone can accept one or two electrons to become a semiquinone or form hydroquinone, respectively. Several synthetic hydroquinones and quinones, such as hydroquinone itself and *tert*-butyl hydroquinone, have been used as antioxidants for rubber and other polymers, as well as for food stuffs [2]. Quinones and related compounds are receiving considerable interest recently, due to their widespread environmental prevalence [3]. *Tert*-butyl hydroquinone (tBHQ), plastoquinone and pyrroloquinoline-quinone have been reported for their antioxidant activity [4-6]. Ubiquinol occurs in human plasma and other tissues, together with its oxidized form, ubiquinone [7-9]. Ubiquinone was shown to be a potent *in vivo* antioxidant under conditions of low oxygen concentrations, which usually occurs in many cellular environments [10]. It has also been suggested that ubiquinol is a more significant antioxidant than tocopherol (vitamin E), carotene or lycopene in inhibiting the oxidation of membrane lipoprotein [11]. The neuroprotective effect of ubiquinone in an experimental model of endotoxemia has been evaluated [12]. Antioxidant activities of ubiquinone were measured in terms of radical and intracellular ROS scavenging activity, lipid peroxidation inhibition, the level of antioxidant enzymes and mitogen activated protein kinase (MAPK) signaling pathway. Antioxidant not only can change antioxidant enzymes and MAPK signaling pathway indirectly, but also serve as a radical quencher, directly. In the latter case, the intrinsic antioxidative activity of hydroquinone could be related to its structure, which carries both electrons and protons; thus, resulting in oxidized and reduced forms of quinones (Eq. 1).



Eq. 1. Electron transfer between quinone and hydroquinone.

Homogentisic acid (2,5-dihydroxyphenylacetic acid), one of the hydroquinone derivatives, is normally metabolized by the enzyme homogentisic acid oxidase (HGO) [13]. When there are mutations in the HGO gene and a deficiency of homogentisate 1,2-dioxygenase, alkaptonuria occurs and it leads to an accumulation of homogentisic acid, ochronosis, and destruction of connective tissue [14]. Autoxidation of homogentisic acid generates oxygen radical, which is assumed to cause alkaptonuric arthritis [15]. Tyrosine is metabolized to homogentisic

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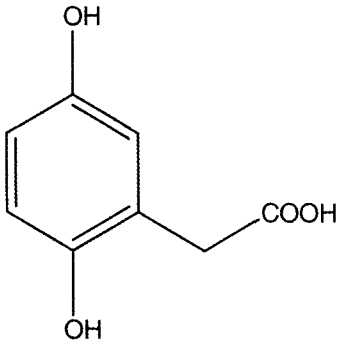

Fig. 1. Chemical structure of homogentisic acid.

acid through the formation of *p*-hydroxyphenylpyruvate and *p*-hydroxyphenyllactic acid. Homogentisic acid has been reported to be mutagenic in bacteria and mammalian cells [16]. Also, homogentisic acid inhibited cell-free protein synthesis [17]. Although alkaptonuria manifests clinically in joints and peripheral tissues, the bulk of tyrosine catabolism and homogentisic acid production occurs in the liver with a minor contribution from the kidneys [18]. Possible factor for toxicity of homogentisic acid in our assumption is not compound itself but the cell line chosen. These reports prompt us to investigate the antioxidant properties of homogentisic acid in WI 38 human lung fibroblast cells.

MATERIALS AND METHODS

Reagents

The homogentisic acid (Fig. 1), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) and Hoechst 33342 were purchased from Sigma Chemical Company (St. Louis, MO, USA). The PeroXOquant™ quantitative peroxide assay kit was purchased from Pierce (Rockford, IL, USA). The other chemicals and reagents were of analytical grade. Primary rabbit polyclonal anti-ERK 2 (42 kDa ERK) and phospho-ERK1/2 (phosphorylated 44 kDa/42 kDa ERK) (Thr 202/Tyr 204) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture

It has been reported that the lung is an organ sensitive to oxidative stress [19,20]. To study the effect of homogentisic acid on the oxidative stress induced by hydrogen peroxide, human lung fibroblasts (WI 38) cells were used and cultured in RPMI 1640, containing 0.1 mM non-essential amino acids, 10% heat-inactivated fetal calf serum, streptomycin (100 μg/mL) and penicillin (100 units/mL).

Intracellular Reactive Oxygen Species (ROS) Measurement and Image Analysis

The DCF-DA method was used to detect the intracellular ROS level [21]. The WI 38 cells were seeded in a 96 well plate at 1×10^5 cells/mL. Sixteen hours after plating, the cells were treated with various concentrations of homogentisic acid, and 30 min later, 1 mM H_2O_2 was added to the plate and the cells incubated for an additional 30 min at 37°C. After the addition of 25 μM of DCF-DA solution, the fluorescence of the 2′,7′-dichlorofluorescein was detected at excitation and emission wavelengths of 485 and 535 nm, respectively, using a Perkin Elmer LS-5B spectrofluorometer.

For image analysis of the production of intracellular ROS, the cells were treated with homogentisic acid, and 30 min later, 1 mM H_2O_2 was added to the plate. After changing the medium, 100 μM of DCF-DA was added to the well, and incubated for an additional 30 min at 37°C. Stained cells were mounted onto a microscope slide in the mounting medium (DAKO, Carpinteria, CA, USA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

DPPH Radical Scavenging Activity

Various concentrations of homogentisic acid were added to a 1×10^{-4} M solution of DPPH in methanol, and the reaction mixture was shaken vigorously. After 1 h, the amount of residual DPPH was determined at 520 nm, using a spectrophotometer [22].

Lipid Peroxidation Inhibitory Activity

Lipid peroxidation was assayed using the thiobarbituric acid reaction [23]. The cells were treated with various concentrations of homogentisic acid. One hour later, 1 mM H_2O_2 was added to the plate, and incubated for further 1 h. The cells were homogenized in ice-cold 1.15% KCl. One hundred μL of the cell lysates was mixed with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid (adjusted to pH 3.5) and 1.5 mL of 0.8% thiobarbituric acid. The mixture was made up to a final volume of 4 mL with distilled water and heated to 95°C for 2 h. After cooling to room temperature, 5 mL of *n*-butanol and pyridine mixture (15:1, v/v) was added to each sample, and the absorbance of supernatant fraction was measured spectrophotometrically at 532 nm.

Cell Viability

The cells were treated with various concentrations of homogentisic acid. One hour later, 1 mM H_2O_2 was added to the plate, and incubated at 37°C for an additional 24 h. Fifty μL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution (2 mg/mL) was then added to each well to attain a total reaction volume of 200 μL. After incubating for 4 h, the plate was centrifuged at 800 × g for 5 min, and the supernatants were aspirated. The formazan crystals formed in each well were dissolved in 150 μL dimethylsulfoxide, and the A_{540} read on a scanning multi-well spectrophotometer [24-26].

Nuclear Staining with Hoechst 33342

The cells were treated with 60 μM of homogentisic acid, and after further incubation for 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, 1.5 μL of Hoechst 33342 (stock 10 mg/mL), a DNA specific fluorescent dye, was added to each well (1.5 mL), and incubated for 10 min at 37°C. The stained cells were then observed under a fluorescent microscope, equipped with a CoolSNAP-Pro color digital camera, in order to examine the degree of nuclear condensation.

Flow Cytometry Analysis

Flow cytometry was performed to determine the apoptotic sub G_1 hypo-diploid cells [27]. The WI 38 cells were placed in a 6 well plate at 1×10^5 cells/mL. Sixteen hours after plating, the cells were treated with 60 μM of homogentisic acid. After a further incubation of 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, the cells were harvested, and fixed in 1 mL of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS, and then incubated for 30 min in the dark at 37°C in 1 mL of PBS containing 100 μg propidium iodide and 100 μg RNase A. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The proportion of sub G_1 hypo-diploid cells was assessed by the histograms generated using the computer program, Cell Quest and Mod-Fit.

Catalase Activity

The WI 38 cells were treated with various concentrations of homogentisic acid for 1 h. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonication twice for 15 sec. Triton X-100 (1%) was then added to the lysates and was incubated for 10 min on ice. The lysates were centrifugated at 5,000 × g for 30 min at 4°C to remove the cellular debris. The protein content of the supernatant was determined by Bradford method [28]. Fifty μg of protein was added to 50 mM of phosphate buffer (pH 7) containing 100 mM (v/v) H_2O_2. The reaction mixture was incubated for 2 min at 37°C, and the absorbance monitored at 240 nm for 5 min [29]. The catalase activity was expressed in units/mg protein, with one unit of enzyme activity defined as the amount of enzyme required to breakdown 1 μM H_2O_2.

Western Blot

The cells were treated with 60 μM of homogentisic acid. The cells were harvested at the indicated times, washed twice with PBS, and then lysed on ice for 30 min in 100 μL of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at 13,000 × g for 15 min. Aliquots of the lysates (40 μg of protein) were boiled for 5 min, and then subjected to electrophoresis in 10% SDS-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then incubated with primary rabbit monoclonal-ERK2 and -phospho ERK1/2. The membranes were further incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugates (Pierce,

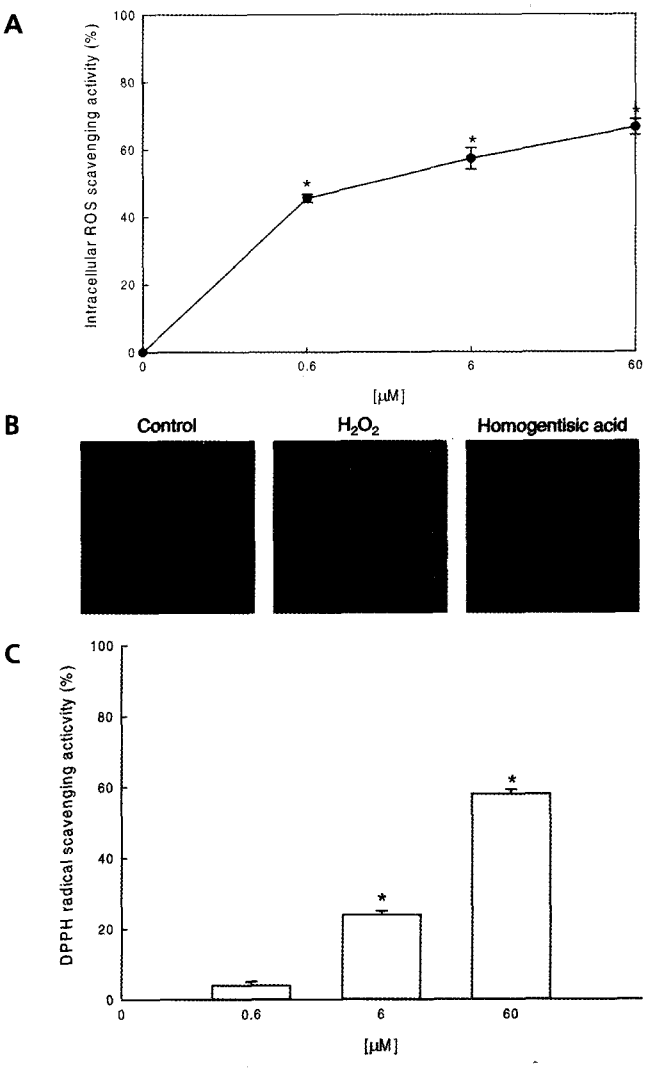

Fig. 2. Effect of homogentisic acid on the scavenging of intracellular ROS and DPPH radicals. The intracellular ROS generated was detected using the DCF-DA method (A) and by confocal microscopy (B). (A) The cells were seeded at 1×10^5 cells/mL and the cells were treated with homogentisic acid, and 30 min later, 1 mM H_2O_2 was added. After the addition of 25 μM of DCF-DA solution, the fluorescence of the 2′,7′-dichlorofluorescein was detected at excitation and emission wavelengths of 485 and 535 nm, respectively. (B) Representative confocal images illustrate the increase in the red fluorescence intensity of DCF produced by ROS in 1 mM H_2O_2 treated WI 38 cells, compared to the control, which show a lowered fluorescence intensity in 1 mM H_2O_2 treated WI 38 cells in the presence of homogentisic acid (original magnification × 400). (C) The amount of DPPH radicals was determined spectrophotometrically at 520 nm. *Significantly different from the control ($p<0.05$).

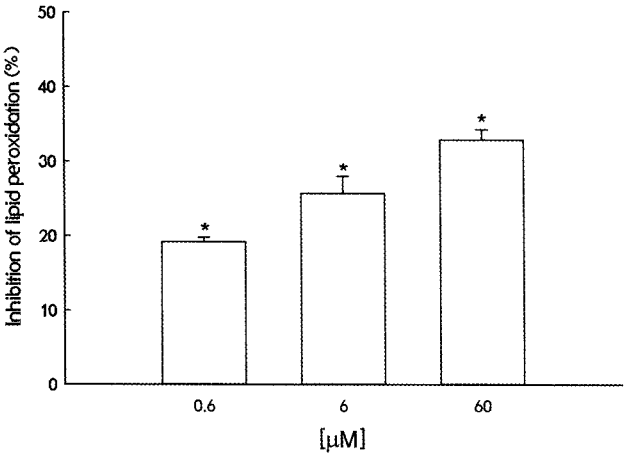

Fig. 3. Effect of homogentisic acid on the inhibition of lipid peroxidation. Lipid peroxidation was assayed by measuring the amount of TBARS formation. The cells were treated with homogentisic acid. One hour later, 1 mM H_2O_2 was and incubated for further 1 h. The cells were homogenized and heated to 95°C for 2 h. Five mL of *n*-butanol and pyridine mixture was added and the absorbance of supernatant fraction was measured spectrophotometrically at 532 nm. *Significantly different from the control ($p<0.05$).

Rockland, IL, USA), and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Measurement of Hydrogen Peroxide

The level of H_2O_2 was determined using PeroXOquant™ quantitative peroxide assay kits, which detect H_2O_2 based on the oxidation of ferrous to ferric ions in the presence of xylenol orange [30].

Statistical Analysis

All the measurements were carried out in triplicate. The results were subjected to an analysis of variance (ANOVA), using the Tukey test, to analyze for the difference. $p<0.05$ were considered significant.

RESULTS

The radical scavenging effects of homogentisic acid on the intracellular ROS and DPPH free radical scavenging activities were measured. The intracellular ROS scavenging activities of homogentisic acid were found to be 45, 57, and 66% at concentrations of 0.6, 6, and 60 μM, respectively (Fig. 2A). In the presence of 2 mM N-acetylcysteine (positive control), there was 88% ROS inhibition (data not shown). As shown in Fig. 2B, the fluorescent intensity of DCF-DA staining was enhanced in the 1 mM H_2O_2 treated WI 38 cells. However, homogentisic acid reduced the intensity of the red fluorescence due to 1

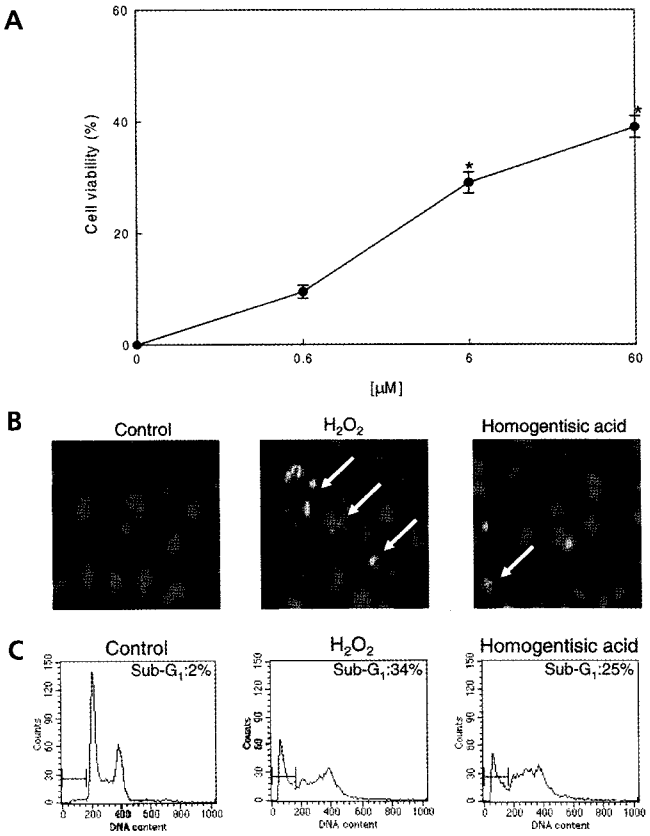

Fig. 4. Protective effect of homogentisic acid on H_2O_2 induced oxidative damage of WI 38 cells. (A) The viability of WI 38 cells was determined using the MTT assay. The cells were treated with homogentisic acid. One hour later, 1 mM H_2O_2 was added to the plate, and incubated at 37°C for an additional 24 h. Fifty μL of MTT stock solution (2 mg/mL) was then added to each well and after incubating for 4 h, the formazan crystals formed in each well were dissolved in dimethylsulfoxide, and the A_{540} read on a scanning multi-well spectrophotometer. (B) Apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining; the apoptotic bodies are indicated by arrows. The cells were treated with 60 μM of homogentisic acid, and after further incubation for 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, 1.5 μL of Hoechst 33342 (stock 10 mg/mL) was added and incubated for 10 min at 37°C. The stained cells were then observed under a fluorescent microscope. (C) Apoptotic sub-G_1 DNA content was detected by flow cytometry after propidium iodide staining. The cells were treated with 60 μM of homogentisic acid. After further incubation of 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, the cells were harvested, and fixed in 1 mL of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS, and then incubated for 30 min in the dark at 37°C in 1 mL of PBS containing 100 μg propidium iodide and 100 μg RNase A. Flow cytometric analysis was performed using a FACSCalibur flow cytometer.

mM H_2O_2 treatment, reflecting a reduction in the ROS generation. The ROS scavenging activity of homogentisic acid was found to be consistent with its DPPH radical

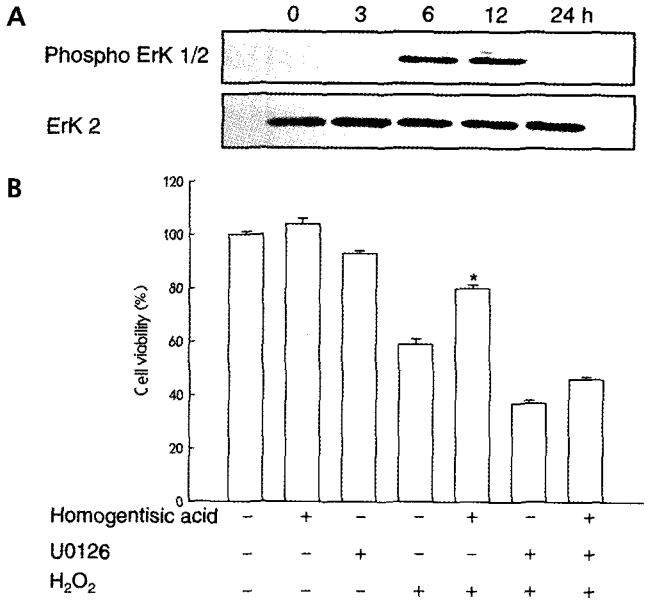

Fig. 5. Effect of homogentisic acid on ERK activation. (A) Aliquots of the lysates (40 μg of protein) were subjected to electrophoresis in 10% SDS-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes and then incubated with primary rabbit monoclonal -ERK2 and -phospho ERK1/2. The membranes were incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugates. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit. (B) After treatment of 10 nM U0126, 60 μM homogentisic acid or/and 1 mM H_2O_2, the viability of WI 38 cells was determined using the MTT assay. *Significantly different from H_2O_2 treated cells ($p<0.05$).

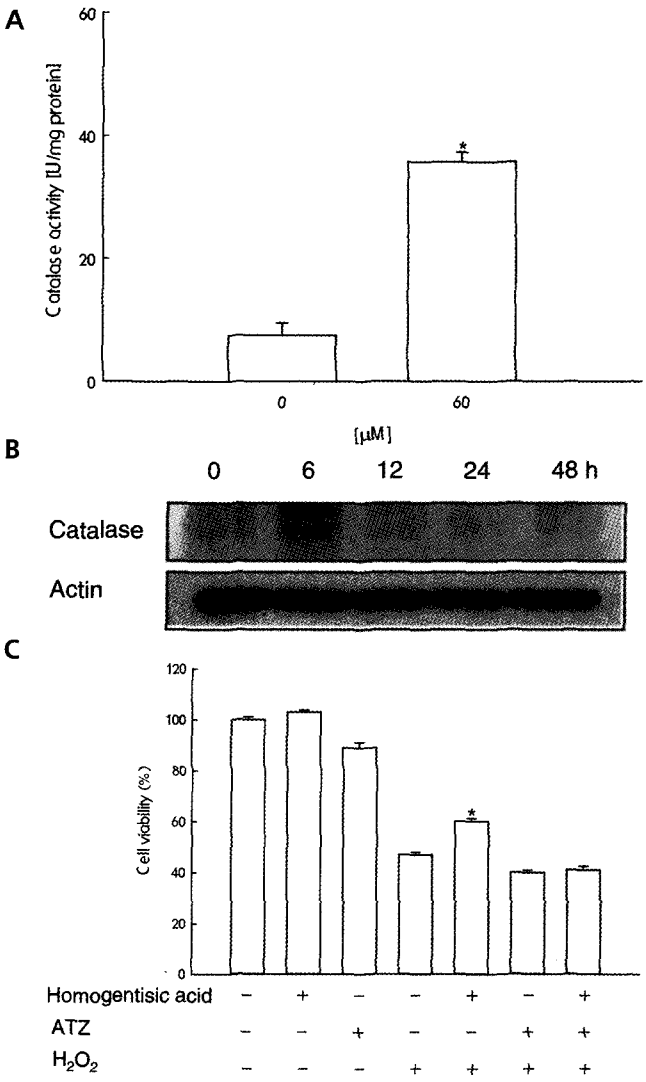

Fig. 6. Effects of homogentisic acid on the activity of catalase. (A) The data represents three experiments, and are expressed as the average enzyme units per mg protein ± S.E. *Significantly different from the control ($p<0.05$). (B) After treatment with 20 mM ATZ, 60 μM homogentisic acid or/and 1 mM H_2O_2, the viability of WI 38 cells was determined using the MTT assay. *Significantly different from H_2O_2 treated cells ($p<0.05$).

scavenging activity (Fig. 2C). The DPPH radical scavenging activities of homogentisic acid were 4, 24, and 58% at concentrations of 0.6, 6, and 60 μM, respectively, compared to the 92% inhibition in the presence of 2 mM N-acetylcysteine. The ability of homogentisic acid to inhibit the lipid peroxidation in 1 mM H_2O_2 treated WI 38 cells was also investigated. The generation of thiobarbituric acid reactive substance (TBARS) was inhibited in the presence of homogentisic acid. The inhibitory effects of homogentisic acid were 19, 26, and 33% at concentrations of 0.6, 6, and 60 μM, respectively (Fig. 3). The protective effect of homogentisic acid on the cell survival in H_2O_2 treated WI 38 cells was also measured. Cells were treated with homogentisic acid, at various concentrations for 1 h, prior to the addition of H_2O_2. The cell viability was determined 24 h later using the MTT assay. As shown in Fig. 4A, treatment with homogentisic acid induced a dose dependent increase in the cell survival rate; 10% at 0.6 μM, 29% at 6 μM, and 39% at 60 μM. In order to study the cytoprotective effect of homogentisic acid on apoptosis induced by H_2O_2, nuclei of WI 38 cells were stained with Hoechst 33342 for microscopy and with propidium iodide for flow cytometric analysis. The microscopic pictures in Fig. 4B showed that the control cells had intact nuclei, but the 1 mM H_2O_2 treated cells show significant nuclear fragmentation, a characteristic feature of apoptosis [31]. However, when the cells were treated with homogentisic acid for 1 h prior to H_2O_2 treatment, a dramatic decrease in nuclear fragmentation was observed. In addition to the morphological evaluation, the protective effect of homogentisic acid on apoptosis was confirmed by flow cytometry. As shown in Fig. 4C, an analysis of the DNA content in the H_2O_2 treated cells revealed an increase of 34% of apoptotic sub-G_1 DNA content, as compared to 2% of apoptotic sub-G_1 DNA content in untreated cells. Treatment with 60 μM of homogentisic acid decreased the apoptotic sub-G_1 DNA content to 25%. The activation of extracellular

Table 1. Generation of H_2O_2 in cell-culture media

	[H_2O_2 μM] Present in RPMI media
None	0.2 ± 0.01
DMSO*	0.1 ± 0.05
Homogentisic acid	0.5 ± 0.10

Homogentisic acid, at a final concentration of 60 μM, was added to the RPMI culture media, and incubated at room temperature for 1 h. Generation of H_2O_2 was then measured using the ferrous iron oxidation-xylenol orange assay. Data are the means ± S.E. *DMSO at the same concentration used to dissolve homogentisic acid.

signal regulated kinase (ERK) is known to induce cell proliferation [32]. To better understand the protective mechanism of homogentisic acid on WI 38 cells, the activation of the ERK protein was examined using western blot analysis with the phospho-ERK specific antibody. As shown in Fig. 5A, after 6 h of homogentisic acid treatment, the phosphorylated ERK was dramatically activated. However, there was no change in the total ERK protein level. To determine the effect of an ERK inhibitor on the protective activity of homogentisic acid against H_2O_2 induced damage in WI 38 cells, the cells were pre-treated for 30 min with U0126 (10 nM), specific inhibitor of ERK kinase, followed by treatment with homogentisic acid for 30 min, and exposured to 1 mM H_2O_2 for 24 h. As shown in Fig. 5B, the U0126 treatment (46%) abolished the protective activity of the homogentisic acid in the 1 mM H_2O_2 damaged cells (80%). In order to investigate whether the radical scavenging activity of homogentisic acid was mediated by the activities of antioxidant enzyme, the activity of catalase in homogentisic acid treated WI 38 cells were measured. Homogentisic acid increased the activity of catalase (Fig. 6A); at a concentration of 60 μM, the activity was 36 U/mg protein, compared to 8 U/mg protein in the control. The results show that the enhancement of the catalase enzyme activity due to homogentisic acid may be associated with the inhibition of the production of ROS. 3-amino-1,2,4 triazol (ATZ) is known as a specific inhibitor of catalase activity [33]. To determine the effect of a catalase inhibitor on the protective activity of homogentisic acid against H_2O_2 induced damage in WI 38 cells, the cells were pretreated with 20 mM of ATZ for 1 h, followed by treatment with homogentisic acid for 30 min, and exposured to 1 mM H_2O_2 for 24 h. As shown in Fig. 6B, ATZ treatment abolished the protective activity of homogentisic acid in H_2O_2 damaged cells. It has been reported that most phenolic compounds interact with commonly used cell culture media to generate H_2O_2 [34]. The generation of low level of H_2O_2 can trigger a rise in antioxidant enzymes. To determine if homogentisic acid generates H_2O_2 in a culture medium, homogentisic acid was added to the cell culture media, to a final concentration of 60 μM and the amount of H_2O_2 generated measured 1 h later using the ferrous iron oxidation-xylenol orange assay. As shown in Table 1, H_2O_2 was scarely detected in the homogentisic acid treated media (<1 μM H_2O_2), suggesting the antioxidant activities in homogentisic acid treated cells were not increased by the H_2O_2 generated in homogentisic acid treated media.

DISCUSSION

Oxidative stress is one of the most important factors in human health, causing inflammation, aging, cancer, arteriosclerosis, hypertension and diabetes [35,36]. Therefore, antioxidants are necessary for maintaining human health [37].

In our present study, it was observed that upon exposure to H_2O_2, homogentisic acid decreased the generation of intracellular ROS and the level of DPPH radicals. Homogentisic acid has a phenol structure. Phenols are electron-rich compounds, which are prone to enter into efficient electron-donation reactions with oxidizing agents, producing phenoxyl radical ($PhO^{\cdot}$) species as intermediates. Phenoxyl radicals are stabilized by resonance delocalization of the unpaired electron to the *ortho* and *para* positions of the ring. In addition to the resonance stability, phenoxyl radicals can also be stabilized by hydrogen bonding with an adjacent hydroxyl group. Phenoxyl radicals also undergo dimerization ("phenol coupling") to produce new C-C or C-O linkages [2]. The intrinsic stability of phenolic structures might be related to the antioxidative activity of homogentisic acid. Homogentisic acid showed a protective effect in H_2O_2 damaged cells. Our results indicated that the inhibition of ROS formation might be important for cytoprotection against oxidative damage. Catalase plays a significant role in effective augmentation of antioxidant defense mechanisms in cells. Homogentisic acid increased the catalase activity, suggesting that the scavenging of ROS may be related to the increased antioxidant activity. In many cell types, the ERK pathway is induced by a variety of extracellular stimuli [38]. The phosphorylation of ERK can phosphorylate cytoplasmic and nuclear targets and participates in a wide range of cellular programs including proliferation, differentiation, and movement [32,39,40]. Phosphorylated ERK was induced in homogentisic acid treated WI 38 cells, and treatment of U0126, a specific inhibitor of ERK kinase, suppressed the protective activity of homogentisic acid in H_2O_2 damaged cells, suggesting that the protective effect of homogentisic acid on cells may also be involved in activating the ERK pathway. Nuclear factor kappa B (NF-kB), a transcription factor, is an important component of intracellular signaling cascades which mediate oxidative survival. Antioxidant enzymes could be potential target molecules mediating antiapoptotic functions of NF-kB against oxidative stress. Sequence analysis of antioxidant enzymes revealed putative binding sites for NF-kB [41-43]. Homogentisic acid increased the activity of antioxidant enzyme and the expression of catalase and the activation of ERK, which is an upstream of NF-kB. Based on these findings, it suggests the possibility that homogentisic acid may involve the NF-kB activation. In addition, JNK and p38 are subfamilies of the MAPK family which constitute important mediators of signal tranduction pathways that serve to

coordinate the cellular response to a variety of extracellular stimuli, through serial phosphorylation cascades [44]. ERK is mainly activated by mitogens and growth factors, while p38 and JNK are activated by the exposure to many environmental stresses (*e.g.* UV, ionizing radiation), resulting in apoptosis [45]. Therefore, it can not exclude the possibility that homogentisic acid may involve the JNK and p38 pathway. The effects of homogentisic acid on cell viability might involve dual actions: the direct action on oxygen radical scavenging, as shown by DPPH radical scavenging, and the indirect action through induction of anti-oxidative enzymes. Homogentisic acid inhibits the formation of hydroxylysine, essentially unique amino acid found in collagen, which is important for the structural function of collagen formation through inhibition of lysine hydroxylase activity [46]. The effective concentration range of homogentisic inhibition was between 0.01 mM and 10 mM. Although high concentrations of antioxidants may decrease oxidative stress, under certain circumstances they may promote the generation of free radical and its related substances, acting as a prooxidant. Even for antioxidants such as carotenoids, ubiquinones, α-tocopherol, flavonoids, and resveratrol analogues, the generation of prooxidant metabolites was suggested [47-51]. For the results of the concentration range (60 μM) we performed, homogentisic acid might act as antioxidant in WI 38 cells, and further experiments should be conducted to determine the antioxidant and prooxidant propeties of homogentisic acid in WI 38 cells.

In conclusion, homogentisic acid exerted intracellular ROS and DPPH radical scavenging activities, promoted cell viability via activation of ERK protein, inhibited H_2O_2 induced apoptosis, and enhanced the effects of antioxidant enzyme.

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