Biomedical Science Letters 2014, 20(2): 56~61
eISSN : 2288-7415

Original Article

Regulation of Nrf2 Mediated Phase II Enzymes by Luteolin in human Hepatocyte

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This study attempted to confirm the antioxidative potential of luteolin against tert-butyl hydroperoxide (t-BHP) induced oxidative damage and to investigate its molecular mechanism related to glutathione (GSH)-dependent enzymes in HepG2 cells. Treatment with luteolin resulted in attenuation of t-BHP induced generation of reactive oxygen species (ROS) and oxidative stress-mediated cell death. In addition, accelerated expression of GSH-dependent antioxidative enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR), and heme oxygenase (HO)-1, as well as strengthened GSH content was induced by treatment with luteolin, which was in accordance with increased nuclear translocation of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), a transcription factor for phase 2 enzymes, in a dose-dependent manner. These results suggest that the cytoprotective potential of luteolin against oxidative damage can be attributed to fortified GSH-mediated antioxidative pathway and HO-1 expression through regulation of Nrf2 in HepG2 cells.

Key Words: Luteolin, Oxidative stress, Glutathione, Heme oxygenase-1, Nuclear factor-erythroid 2 p45-related factor 2

INTRODUCTION

Oxidative stress initiates overproduction of reactive oxygen species (ROS) that damage lipids, proteins, and DNA, which is implicated in the pathogenesis of several human diseases, including atherosclerosis, cancer, neuro-degenerative diseases, and aging (Thannickal and Fanburg, 2000). In addition, oxidative stress plays a critical role in the process of hepatic disorders because liver is the target organ of xenotoxic agents capable of generating ROS (Jaeschke et al., 2002). Therefore, many natural resources for scavenging free radicals have been identified and have been proposed as therapeutic agents to counteract liver damage (Vitaglione et al., 2004). Flavonoids, plant poly-phenolic compounds abundant in fruits and vegetables, have been studied for their potent antioxidative capacity (Jang et al., 2008). Among them, luteolin (3',4',5,7-tetrahydroxyflavone) scavenged lipopolysaccharide (LPS) induced formation of ROS in RAW 264.7 cells and restored antioxidative enzyme activities in an azoxymethane (AOM)-induced colon carcinogenic animal model (Ashokkumar and Sudhandiran, 2008; Park et al., 2011).

HepG2 cells, a human hepatocellular carcinoma cell line, are considered a good *in vitro* model for study of xenobiotic metabolism. Although HepG2 cells are a class of cancer cell line, they possess a variety of functions that are exhibited in normal human hepatocytes (Krithika R et al., 2009). In particular, HepG2 cells exhibit various kinds of enzyme activities, including phase I, II, and antioxidative enzymes, which indicates that they are a good model for investigation of many biological reactions, such as antioxidative, cyto-protective, genotoxic, and antigenotoxic effects induced by xenobiotic compounds (Knasmuller et al., 2004; Mersch-Sundermann et al., 2004).

The aim of this study was to analyze the protective

*Received: February 7, 2014 / Revised: April 4, 2014
Accepted: April 16, 2014

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activity of luteolin against oxidative damage and its underlying molecular mechanism related to nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)-mediated antioxidative cascade in HepG2 cells.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and glutamine were obtained from Invitrogen (Carlsbad, CA, USA). Luteolin (purity ≥98.0%), 2',7'-dichlorofluorescin diacetate (DCFH-DA), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technology (Danvers, MT, USA), and Enzo Life Sciences (Farmingdale, NY, USA). Polyvinylidene Fluoride (PVDF) membrane was obtained from Bio-Rad (Hercules, CA, USA). All other chemicals were of the highest commercial grade available.

Cell culture and treatment

The HepG2 cell line was obtained from the American Type Culture Collection (HB-8065; Rockville, MD, USA) and cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. To investigate the question of whether luteolin can attenuate t-BHP induced oxidative damage, optimized conditions for oxidative damage-mediated cell death in HepG2 cells should be determined. Cells were treated with various concentrations of t-BHP for 0, 1, 3, 6, 12, and 24 hr. Increased concentration of t-BHP resulted in elevated cytotoxicity, as shown by decreased cell viability in a dose and time dependent manner. Among various concentrations, treatment with 0.5 mM of t-BHP for 3 hr provoked approximately 30% cell death and this condition was selected for the following experiments (Song and Park, 2013).

Cell viability assay

Cell viability was determined using a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay purchased from Promega (Madison, WI, USA). Cells were incubated with MTS for 1 hr and quantified by measurement at OD_{490}.

Glutathione (GSH) content

GSH was measured using an enzymatic recycling procedure described by Tietze (Tietze, 1969), in which GSH is sequentially oxidized by 5,5U-dithiobis (2-nitrobenzoic acid) and reduced by NADPH in the presence of GSH reductase.

ROS formation assay

ROS scavenging activity was measured using the cell permeable fluorescent dye DCFH-DA. HepG2 cells were stained with 50 μM of DCFH-DA for 2 hr. The cells were then preincubated with luteolin and subsequently incubated with t-BHP (0.5 mM) for 1 hr for induction of oxidative stress. Dye fluorescence was measured in a multi-detection reader (Synergy HT, Biotek, USA) at an excitation and emission wavelength of 485 nm and 530 nm, respectively.

Western blot analysis

Total cell lysate was obtained from protein extraction solution (M-PER, Pierce Biotechnology, Rockford, IL, USA). Nuclear and cytoplasmic extracts were prepared using an NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology) according to the manufacturer's instructions. Protein samples (50 μg) from each lysate were separated on a 10% SDS-polyacrylamide gel and electro-transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in TBST solution for 2 hr at room temperature. The membranes were then incubated with primary antibodies in blocking buffer at 4℃ overnight. After washing, the membranes were further incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody for 2 hr at room temperature. The blot was developed using enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology). Data were quantified using the Gel Doc EQ System (Bio-Rad). All signals were normalized to protein expression levels of the β-actin and expressed as a ratio.

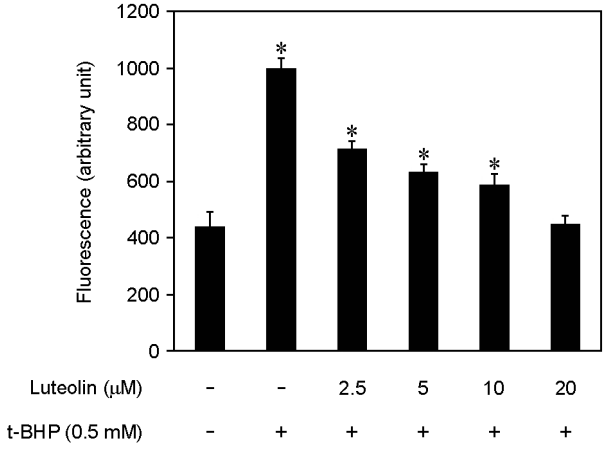

Fig. 1. Luteolin scavenged t-BHP-induced ROS generation in HepG2 cells. HepG2 cells were stained with 50 μM of DCFH-DA for 2 hr. Then, the cells were pre-incubated with either luteolin for 2 hr and subsequently incubated with t-BHP (0.5 mM) for 30 min to induce the ROS generation. The data represent the mean ± SD of triplicate experiments. Significant differences between untreated control and t-BHP treated groups were evaluated by unpaired Student's *t*-test. **P*<0.05 vs. untreated group.

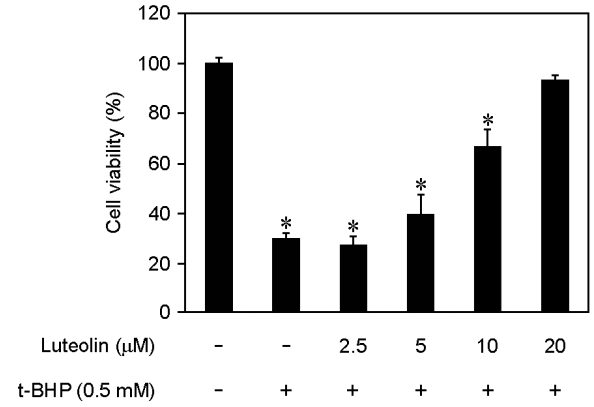

Fig. 2. The antioxidative potential of luteolin against the t-BHP-induced oxidative damage in HepG2 cells. HepG2 cells were treated with indicated concentrations of luteolin (0, 2.5, 5, 10 or 20 μM) for 12 hr. The untreated and the luteolin treated cells were exposed to 0.5 mM t-BHP for 3 hr. The data represent the mean ± SD of triplicate experiments. Significant differences between untreated control and t-BHP treated groups were evaluated by unpaired Student's *t*-test. **P*<0.05 vs. untreated group.

Statistical analysis

The data were expressed as mean ± standard deviations (SD). Statistical analyses were performed using SPSS version 13.0 (SPSS Institute, Chicago, IL, USA). The values were compared with the control using analysis of variance (ANOVA) followed by unpaired Student's *t*-test. *P* values less than 0.05 ($P<0.05$) were considered significant.

RESULTS AND DISCUSSION

Use of various phytochemicals for scavenging free radicals is considered a reasonable strategy for protection of the liver from xenobiotic-induced oxidative damage. Therefore, recent investigations have focused on finding new natural dietary compounds possessing antioxidative activity (Masella et al., 2005). Although the antioxidative capacities of many phytochemicals have been proven, their molecular mechanisms are not sufficiently understood. Lima *et al*. compared antioxidative activity of five phenolic compounds, including luteolin, luteolin-7-*O*-glucoside, quercetin, rosmarinic acid, and caffeic acid through the protective potential against t-BHP induced cell death in HepG2 cells (Lima et al., 2006). Therefore, this study attempted to verify the protective activity of luteolin against t-BHP induced oxidative damage and to further investigate the molecular mechanism of the Nrf2-mediated GSH pathway and HO-1 expression in HepG2 cells.

ROS triggers lipid peroxidation and subsequent cell injury; therefore, increased ROS production could be a primary factor for hepatotoxicity (Nakagawa Y et al., 2010). In this study, t-BHP, an organic hydroperoxide, was applied for induction of oxidative damage in HepG2 cells. t-BHP is metabolized in hepatocytes *via* two distinct pathways. One is involved with cytochrome P450, which leads to formation of toxic peroxyl and alkoxyl radicals, and initiates lipid peroxidation. Oxidative damage caused a sharp increase of ROS generation accompanied by accelerated cytotoxicity, which was significantly ($P<0.05$) attenuated by treatment with luteolin in a dose dependent manner (Fig. 1 and 2). These results suggest a potential role of luteolin as a hepatoprotective agent against oxidative damage as a potent ROS scavenger.

The other is a detoxification process in which t-BHP reacts with GSH peroxidase (GPx), which produces tert-butyl alcochol and oxidizes GSH (Hwang YP et al., 2011). GSH, one of the primary defense systems against oxidative

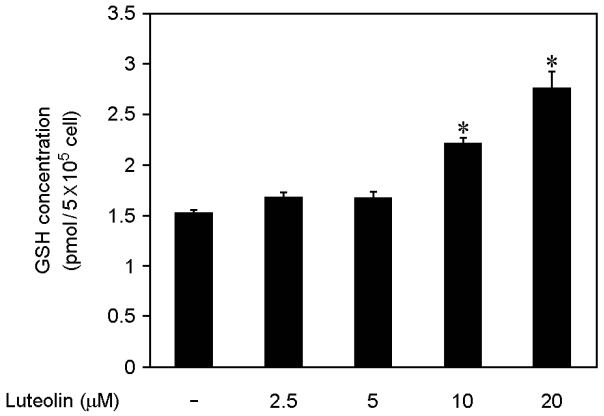

Fig. 3. Luteolin restored intracellular GSH concentration in HepG2 cells. HepG2 cells were treated with and without indicated concentrations of luteolin for 20 hr. Data represent the mean ± SD of triplicate experiments. Significant differences between untreated control and luteolin treated groups were evaluated by unpaired Student's *t*-test. **P*<0.05 vs. untreated group.

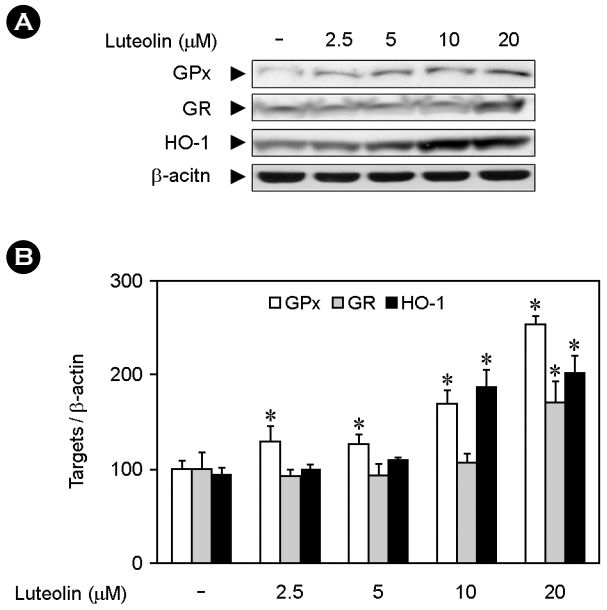

Fig. 4. Luteolin induced GPx, GR and HO-1 protein expression in HepG2 cells. (A) Luteolin induced GPx, GR and HO-1 protein expression as a function of concentration. HepG2 cells were treated for 4 hr with luteolin at the indicated concentrations (0, 2.5, 5, 10 or 20 μM). Each protein expression was analyzed by Western blot analysis. The data are representative of three independent expreiments. (B) The relative induction of GPx, GR and HO-1 expression was quantified by densitometry. β-actin was used as an internal control. The data represent the mean ± SD of triplicate experiments. Significant differences between untreated control and luteolin treated groups were evaluated by unpaired Student's *t*-test. **P*<0.05 vs. untreated group. GPx, glutathione peroxidase; GR, glutathione reductase; HO-1, heme oxyganse-1.

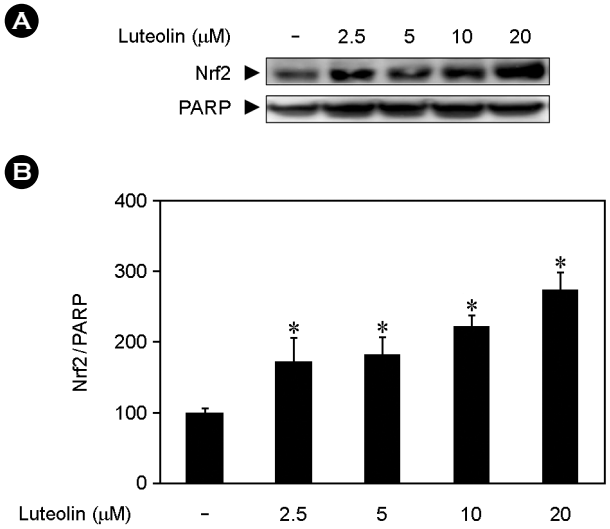

Fig. 5. Luteolin induced nuclear translocation of Nrf2, transcription factor for phase II enzymes, in HepG2 cells. (A) Luteolin induced nuclear translocation of Nrf2 as a function of concentration. HepG2 cells were treated for 4 hr with luteolin at the indicated concentrations (0, 2.5, 5, 10 or 20 μM). Nrf2 nuclear translocation was determined from a nuclear extract by Western blot analysis. The data are representative of three independent expreiments. (B) The relative induction of Nrf2 translocation was quantified by densitometry. PARP was used as an internal control. The data represent the mean ± SD of triplicate experiments. Significant differences between untreated control and luteolin treated groups were evaluated by unpaired Student's *t*-test. **P*<0.05 vs. untreated group. Nrf2, nuclear factor-erythroid 2 p45-related factor 2; PARP, poly (ADP-ribose) polymerase.

stress, plays a role as an essential intracellular reducing agent for maintenance of thiol groups on intracellular proteins and for antioxidant molecules. In addition, glutathione reductase (GR) regulates cellular GSH homeostasis by conversion of oxidized glutathione (glutathione disulfide, GSSG) to reduced form (GSH) (Rebrin I et al., 2011). As shown in Fig. 3, treatment with luteolin resulted in a significantly (P<0.05) fortified intracellular GSH concentration in a dose dependent manner, suggesting that scavenging capacity of t-BHP-induced radicals was elevated in HepG2 cells.

Nrf2 is known to play a critical role in protection of cells against oxidative stress (Kensler et al., 2007). In response to oxidative stress, Nrf2, anchored by a Keap1, a cytosolic repressor, is freed from Keap1 and is translocated into the nucleus, and then binds to antioxidant-response elements (AREs) in the promoters of target genes (Lee and Surh, 2005). This leads to transcriptional induction of GSH-

dependent antioxidant enzymes such as GPx2, GR, and heme oxygenase (HO-1) (Harvey et al., 2009; Kensler et al., 2007). HO-1 is the rate limiting enzyme which catalyzes heme to biliverdin, carbon monoxide, and free iron. By-products of HO-1 catabolism, CO and biliverdin/bilirubin, have shown protective effects against oxidative and inflammatory stimuli (Farombi and Surh, 2006). CO, a gaseous by-product of heme metabolism, contributes to attenuation of pro- inflammatory processes. Bilirubin was a potent scavenger of peroxyl radicals *in vitro* as strong as α-tocopherol and protected cells from hydrogen peroxide-induced oxidative damage (Baranano et al., 2002; Mancuso C., 2012). Western blot analysis was applied for investigation of the molecular mechanisms of luteolin-induced antioxidative potential through modulation of Nrf2 in HepG2 cells. As shown in Fig. 4 and 5, luteolin induced expression of GSH dependent antioxidative enzymes and HO-1 in accordance with the accelerated nucleic translocation of Nrf2.

In conclusion, these results suggest that luteolin treatment strengthens antioxidative potential by GSH restoration. In addition, increased expression of GSH-dependent antioxidative enzymes and HO-1 could be regulated by accumu-lated nuclear translocation of Nrf2 in HepG2 cells. Therefore, luteolin, which exhibited potent antioxidative properties, might be proposed as a potential therapeutic agent against liver damage.

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