PROTECTIVE EFFECTS OF GREEN TEA POLYPHENOLS ON HUMAN HepG2 CELLS AGAINST OXIDATIVE DAMAGE OF FENOFIBRATE

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Abstract—The aim of this work was to investigate the protective effects of green tea polyphenols on the cytotoxic effects of hypolipidemic agent fenofibrate (FF), a peroxisome proliferator (PP), in human HepG2 cells. The results showed that high concentrations of FF induced human HepG2 cell death through a mechanism involving an increase of reactive oxygen species (ROS) and intracellular reduced glutathione (GSH) depletion. These effects were partially prevented by antioxidant green tea polyphenols. The elevated expression of PP-activated receptors α (PPARα) in HepG2 cells induced by FF was also decreased by treatment with green tea polyphenols. In conclusion, this result demonstrates that oxidative stress and PPARα are involved in FF cytotoxicity and green tea polyphenols have a protective effect against FF-induced cellular injury. It may be beneficial for the hyperlipidemic patients who were administered the hypolipidemic drug fenofibrate to drink tea or use green tea polyphenols synchronously during their treatment. © 2003 Elsevier Inc.

Keywords—Green tea polyphenols, Fenofibrate, Peroxisome proliferator-activated receptor (PPAR), HepG2, Oxidative stress, Antioxidant, Free radicals

INTRODUCTION

Peroxisome proliferators (PPs) are a structurally diverse group of chemicals that include fibrate hypolipidemic agents, steroids, herbicides, and other chemicals [1]. They bind to and activate specific transcription factors belonging to the nuclear hormone receptor superfamily, the peroxisome proliferator-activated receptors (PPARs) [2]. Among the three principal isoforms so far identified (PPARα, PPARβ, and PPARγ), PPARα seems to mediate the hypotriglyceremic effect of fibrates by inducing high rates of mitochondrial and peroxisomal β-oxidation in liver, kidney, heart, and muscle and by decreasing the plasma concentration of triacylglycerol-rich lipoproteins [3]. PPARα also has been found to mediate the activity of PPs in mice [4]. Fenofibrate (FF), a member of the fibrate class of lipid-modifying drugs, is extensively used in many countries. FF has been identified as a PP in rodents and research has shown that prolonged exposure to PPs causes an increased incidence of liver tumors in mice and rats [5]. On the other hand, our recent study showed that a high concentration of FF had cytotoxic effects in human HepG2 cells [6].

Tea (Camellia sinensis) is one of the most popular beverages consumed worldwide. Green tea polyphenols are the major water-soluble components in green tea liquor. The polyphenols are the most significant group of tea components, especially certain catechins. The major tea catechins are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG). During the last decade, numerous in vitro and in vivo studies suggested that tea and tea polyphenols had strong antioxidant activity [7–10]. Tea catechins are strong scavengers of superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide produced by various chemicals. They chelated with metals because of the catechol structure and prevented metal-catalyzed free radical formation [11]. Meanwhile, tea catechins can spare urate, β-carotene, and vitamins C and E, contributing to the overall antioxidant protection mechanism of the cell [12].

The objective of the current study was to examine the protective effects of green tea polyphenols in cell injury
induced by FF and to characterize the mechanisms involved. The experimental results showed that incubation of HepG2 cells with a cytotoxic concentration of FF (100 μM) induced GSH depletion and an accumulation of intracellular ROS. These effects were partially prevented by antioxidant green tea polyphenols. Moreover, the elevated transcript level of PPARα in HepG2 cells induced by FF was also decreased by treatment with green tea polyphenols.

MATERIALS AND METHODS

Reagents

DMEM (for cell cultures), fetal bovine serum (FBS), HEPES, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) were purchased from Gibco BRL (Gaithersburg, MD, USA). 2, 7-dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA), and trypsin and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fenofibrate was provided by Laboratoires Fournier (Dijon, France). All other chemicals made in China were of analytical grade. Green tea polyphenols (from the Tea Department of Zhejiang University) used in this study contained 99% catechin derivatives, among which the major components were 50% EGCG, 22% ECG, 18% EGC, and 10% EC (Fig. 1). The average molecular weight of green tea polyphenols was calculated according to the percentage of the major component.

Cell culture

HepG2 cell line (HB8065; American Type Culture Collection, Rockville, MD, USA) is a human hepatoblastoma cell line with a wide variety of liver-specific metabolic responses to different kinds of drugs [13]. HepG2 cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 2 mM L-glutamine in a humidified atmosphere with 5% CO2 at 37°C. The cells were subcultured every 5–7 d at 1:3 split ratios. Cells were used for experiments within eight passages to ensure cell line stability. Medium was changed every 2 d. Stocks of cells were routinely frozen and stored in liquid N2. PPARα activator was dissolved in DMSO as 1000-fold stock solutions and diluted 1000-fold into culture medium. Control cells received an equivalent amount of DMSO.

When antioxidants were tested, the cells were simultaneously incubated in the presence of FF and green tea polyphenols. Different concentrations of green tea polyphenols were previously incubated for 30 min before FF addition and maintained during FF exposure.

Determination of cell viability evaluated as mitochondrial activity

Cell viability evaluated as mitochondrial activity was quantified by measuring dehydrogenase activity retained in the cultured cells, using MTT assay. The assay is based on the ability of living cells to convert dissolved MTT into insoluble formazan; therefore, the amount of formazan produced is proportional to the number of living cells. The cells were plated at a density of 1.3 × 10^5/cm^2 in 96-well plates. After drug treatments, the cells were incubated with 1 mg/ml MTT in DMEM for 4 h at 37°C. Then the MTT-containing medium was removed and the intracellular formazan product was solved in DMSO for quantification at A560.

Measurement of reactive oxygen species (ROS)

The intracellular ROS production was measured using a nonfluorescent compound 2′,7′-dichlorofluorescin diacetate (DCFH-DA) by the method of Bass and coworkers [14], with some modification. This method measures the formation of hydrogen peroxide generated by an oxidative metabolic burst. Viable cells can deacetylate DCFH-DA to 2′,7′-dichlorofluorescin (DCFH), which is not fluorescent. This compound reacts quantitatively with oxygen species within the cell to produce the fluorescent dye 2′,7′-dichlorofluorescin (DCF), which remains trapped within the cell and can be measured to provide an index of intracellular oxidation. For the study of concentration-dependent effects of FF on ROS production in HepG2 cells after 24 h incubation, the cells were collected and loaded with 20 μM DCFH-DA (dis-
solved in DMSO) for 30 min at 37°C. After washing out the excess probe, the cells were transferred to a fluorometer cuvette, and the fluorescence was recorded at 490 and 530 nm excitation and emission (bandpass 2.5 nm), respectively, in a Perkin-Elmer LS 50B spectrophotofluorimeter (Perkin-Elmer Inc., Oak Brooke, IL, USA).

For the study of transient change of ROS right after exposure to FF, the cells were harvested and replated at subconfluent densities into 35 mm² culture dishes with a glass bottom. After 24 h in these dishes, the cells (2 × 10⁵/ml) were incubated with 20 μM DCFH-DA for 30 min at 37°C. After the loading period, the cells were washed twice with PBS, and ROS measurements were performed using AquaCosmos (version 2.00) software provided by Hamamatsu Photonics K.K. (Hamamatsu, Japan). The cells were visualized with a 40× objective on a Nikon 300 microscope (Nikon Inc., Melville, NY, USA), and ROS level was expressed as a percentage of baseline ROS for each cell.

**Determination of the intracellular GSH content**

The samples for measuring GSH were processed according to the methods published before [15]. Briefly, the cells were seeded at 5000 cells/cm² in Falcon 6-well plates. After washing twice with phosphate buffer (pH 7.4), the cultured monolayers were scraped into 1.0 ml of 0.1 M phosphate buffer (pH 8.3) containing 5 mM EDTA. The cell suspension was deproteinized with 200 μl 20% TCA, homogenized by gentle sonication at 0°C, and then centrifuged for 10 min at 5000 × g. The final assay mixture contained 50 μl of the diluted supernatant, 0.9 ml of phosphate EDTA buffer (pH 8.3), and 50 μl (final 50 μg/ml) of the o-phthalthaldehyde solution. After thorough mixing and incubation at room temperature for 30 min, the fluorescence was read at an emission wavelength of 420 nm and an excitation wavelength of 350 nm. This assay was suitable for routine GSH measurements and was determined to be comparable to the HPLC method in terms of its specificity and sensitivity for GSH.

**Determination of PPARα expression**

Total RNA was isolated from Hep2 cells using RNA Isolation kit (Gibco BRL), and the mRNA level of PPARα was determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis, as described previously [16]. An aliquot of 1 μg total RNA from each sample was reverse transcribed to cDNA using a First-Strand cDNA Synthesis kit (Gibco BRL). A standard 25 μl polymerase chain reaction (PCR) contained 1 μl of the reverse transcriptase reaction, 0.2 mmol/l dNTP, 1.5 mmol/l MgCl₂, 1× assay buffer B, 1.5 U Taq polymerase (Promega, Madison, WI, USA), 30 pmol forward primer, and 30 pmol reverse primer (Life Technologies Inc., Grand Island, NY, USA). The primers of PPARα were designed according to the published sequences: 5’-GTG GCT ATA TTG TCG TGT G-3’ (upstream) and 5’-GAA GGT GTC ATG ATG GGT G-3’ (downstream). The primers of β-actin were used as an internal standard to normalize the results. Aliquots (10 μl) of the PCR reaction were elecrophoresed on a 1.4% agarose gel. PCR products were visualized with ethidium bromide. Amplification of each gene yielded a unique band of the expected size (PPARα: 328 bp; β-actin: 545 bp).

**Quantifying triglyceride content**

Cells harvested in cell counting solution were sonicated prior to the addition of 5% (v/v) Triton X-100 to ensure homogenous lipid distribution in all samples [17]. Triglyceride (TG) content was measured using a colorimetric assay that quantifies the glycerol content of the samples (Zhong Sheng Co., Beijing, China). This assay involves the enzymatic hydrolysis of TG by lipases to free fatty acids and glycerol. The glycerol moiety, through a series of oxidation-reduction reactions, then associates with 3,5 dichloro-2-hydroxybenzene sulfonate and 4-aminantipyrine to produce a red-colored dye. The absorbance of this dye is proportional to the concentration of TG present in each sample. The absorbance quantified at 520 nm following these reactions.

**Statistical analysis**

Experimental data were analyzed by Student’s t-test and expressed as mean ± SD. A probability value of p < .05 was considered statistically significant.

**RESULTS**

**Protective effect of green tea polyphenols on HepG2 cells against FF injury**

The effect of FF on cell viability was evaluated by determining the percentage of MTT reduction upon incubation of HepG2 cells with increasing FF concentrations in the range of 0–250 μM. As shown in Fig. 2A, FF elicited a concentration- and time-dependent reduction in cell viability. The protective effect of different concentrations of green tea polyphenols is shown in Fig. 2B. It can be found that cell viability was greatly rescued when pretreated with 200 μM green tea polyphenols.

**The effect of green tea polyphenols on the ROS overproduction induced by FF**

To clarify the involvement of oxygen radical in damaged HepG2 cells induced by FF, the accumulation of ROS after FF exposure was measured by a converting reaction of DCFH-DA to DCF. Results show that FF
induced ROS production after the cells were treated with FF for only a few minutes. The ROS level was two times higher than at the initiation point and, thereafter, reached a plateau (Fig. 3A). After 24 h of incubation with FF, there was a concentration-dependent increase in ROS production (Fig. 3B). The generation of ROS increased dramatically when the cells were treated by FF in the concentration of 100–250 μM. A decrease in ROS production was observed in the cells pretreated with green tea polyphenols (Fig. 3C); 200 μM of green tea polyphenols almost inhibited the ROS overproduction in HepG2 cells induced by 150 and 200 μM FF.

The effect of green tea polyphenols on the GSH depletion induced by FF

GSH is an important cellular antioxidant. To determine the effect of FF on the redox status of cells, GSH level was evaluated in the cells treated with FF (Fig. 4A).
In contrast to the increase of ROS, GSH decreased dramatically when the cells were treated with FF in the concentration of 100–300 μM. This result and ROS accumulation suggest that redox imbalance or oxidative stress was induced in the cells treated with FF. When pretreated with 200 μM green tea polyphenols, GSH level was greatly increased in the cells treated with 150 and 200 μM FF (Fig. 4B).

The effect of green tea polyphenols on transcript levels of PPARα

The RT-PCR products from total RNA of HepG2 cells analyzed by electrophoresis with ethidium bromide in 1.4% agarose gel are shown in Fig. 5 (representative of three separate experiments). A single band with a predicted size (328 bp) was detected in RT-PCR products from RNA of the HepG2 cells. The levels of PPARα mRNA in FF-treated cells, determined by densitometric analysis, were 1.8- ± 0.4-fold higher than in untreated cells. When preincubated with green tea polyphenols, the elevated expression of PPARα induced by FF was decreased and the levels of PPARα mRNA were 1.3- ±
0.2-fold higher than in untreated cells. The ratio of the three groups was 1:1.8:1.3.

The effect of FF and green tea polyphenols on triglyceride content

The triglyceride content in control cells was significantly higher than that in the cells treated with FF (100 μM) and cells treated with FF (100 μM) together with green tea polyphenols (200 μM), and there was no significant difference between the two latter groups. This indicates that triglyceride synthesis in HepG2 cells can be inhibited by FF treatment and this effect is not hindered by green tea polyphenols (Fig. 6).

**DISCUSSION**

The antioxidant activities of tea catechins have been examined by various methods in vitro and in vivo. In the lipoprotein oxidation model that simulates the oxidation of low-density lipoproteins (LDL) responsible for atherosclerosis, tea catechins also exhibited powerful antioxidant activity that was 20 times more potent than vitamin C [18,19]. It was reported that green tea polyphenols exerted inhibitory effects on LDL oxidation both in vitro and in vivo, leading to a reduction in the risk of cardiovascular disease [20]. The hypolipidemic effect of green tea polyphenols was also reported [21].

FF, a member of the fibrate class of lipid-modifying drugs, has been used extensively in many countries since 1975. The molecular mechanism underlying the effect of fibrates has been elucidated [22]. Fibrates bind to and activate specific transcription factor PPARs. These receptors form heterodimer with retinoid X receptor (RXR) and bind to peroxisome proliferator response element (PPRE) in the promoter region of PP-responsive genes, whose transcription rate is consequently regulated. PPARs regulate lipid metabolism, glucose homeostasis, and cell proliferation, thereby integrating nutritional, metabolic, and environmental stimuli within the regulatory network [23]. PPARα seems to mediate the hypotriglyceridemic effect of fibrates and the activity of PPs in mice. FF has been identified as a PP in rodents, and studies have shown that prolonged exposure to PPs caused an increased incidence of liver tumors in mice and rats [24]. In contrast to the tumor-promoting effects in rodents, a growth-inhibitory effect of FF in human HepG2 cells was found in this work (Fig. 2A). This effect was greatly prevented by 200 μM green tea polyphenols (Fig. 2B).

PPARs are also members of the redox-regulated transcription factors. Transcription factors directly influenced by ROS include nuclear factor κB (NF-κB), activator protein-1 (AP-1), specificity protein (Sp1), PPARs,
and other members of the nuclear receptor superfamily [25]. Our study showed that PPARα expression was enhanced in HepG2 cells following FF treatment, and the elevated expression of PPARα induced by FF was decreased when preincubated with green tea polyphenols (Fig. 5). This activation and decrease may be redox-associated and may be seen as a negative feedback to regulate redox imbalance. This also suggests that the cellular injury by FF and the protective effect of green tea polyphenols against the injury were regulated by PPARα.

As the stimulation of ROS and the intracellular depletion of GSH constitute the main cytotoxic mechanisms of a medicine, we investigated whether FF induced ROS production and/or GSH depletion in a well-adapted cellular model for in vitro cytotoxic mechanisms. Moreover, the protective effect of green tea polyphenols was explored on the cell against FF cytotoxicity. Our results show that, in HepG2 cells, FF induced ROS production and GSH depletion in a concentration-dependent manner (Figs. 3B and 4A), and the ROS overproduction and GSH depletion were almost inhibited when cells were preincubated with 200 μM green tea polyphenols (Figs. 3C and 4B). These data lead to the conclusion that oxidative stress is one of the mechanisms of FF cytotoxicity and green tea polyphenols have protective effects against such oxidative injury.

The hypolipidemic effects in pharmacological doses and cytotoxic effects in high concentrations of FF may be carried out in different pathways [26]. Our study found that the cytotoxic effects of FF were related to redox as well as PPARα. A high concentration of FF may exert its cytotoxic effects in a way different from that of the hypolipidemic effects. Hence, it seems reasonable that the green tea polyphenols had protective effects on human HepG2 cells against oxidative damage of a cytotoxic dose of FF, whereas the green tea polyphenols didn’t hinder the hypolipidemic action of FF.

In summary, the present study clarified the protective effects of green tea polyphenols on cell injury induced by a high concentration of FF in human hepatoblastoma cells, and some relevant mechanisms were addressed. Although long-term administration of FF caused liver cancer in susceptible species, a high concentration of FF was found to have the effect of inducing cell death on human liver cells with the involvement of oxidative stress. These cytotoxic effects were partially prevented when the cells were preincubated with green tea polyphenols. The results of our study, together with some previous reports, suggest that it may be beneficial for the hyperlipidemic patients who were administered the hypolipidemic drug fenofibrate to drink tea or use green tea polyphenols synchronously during their treatment.

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**ABBREVIATIONS**

DCF—2’-7’-dichlorofluorescein
DCFH—2’-7’-dichlorofluorescein diacetate
FF—fenofibrate
GSH—reduced form of glutathione
GTP—green tea polyphenols
MTT—(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliunbromide)
PP—peroxisome proliferator
PPARs—peroxisome proliferator-activated receptors
ROS—reactive oxygen species