

RESEARCH COMMUNICATION

Cytoprotective Activity of a Trans-chalcone Against Hydrogen Peroxide Induced Toxicity in Hepatocellular Carcinoma (HepG2) Cells

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Abstract

Dietary flavonoids have attracted attention as chemopreventive agents. Chalcones are abundantly present in nature starting from ferns to higher plants. Chemically 1,3-diphenyl-2-propen-1-ones, these are often cytotoxic in vitro. The cellular defense system (including glutathione, glutathione-related enzymes, and antioxidant and redox enzymes) plays a crucial role in cell survival and growth in aerobic organisms. In the present study, we aimed to evaluate the modulatory effect of trans-chalcone on protection from oxidative stress caused by hydrogen peroxide (H₂O₂) in hepatocellular carcinoma (HepG2) cells. Cell growth was evaluated by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Sub-toxic concentrations of compound (20uM) increased cell survival and a decreased lipid peroxidation. The drug also decreased the H₂O₂ induction of glutathione related enzymes. Our results support the efficacy of trans-chalcone in offering protection against oxidative stress.

Keywords: Hepatocellular carcinoma - tTrans-chalcone - hydrogen peroxide toxicity - lipid peroxidation - HepG2 cells

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Introduction

As we all know the prevention of chronic diseases is a more effective strategy than their treatment, reducing the risk of diseases such as cardiovascular disease and cancer is a subject of great interest for researchers (Liu, 2003). The most potential targets for functional food science are redox and antioxidant systems. For the same reason, nowadays functional foods are aimed at boosting intakes in order to reduce the disease associated with oxidative stress. Bioflavonoids found in fruits and vegetables, have been identified as the therapeutic bioactive compounds providing the health benefits. There has been quite increasing attention over last ten years dedicated to chalcones as they have found to have diverse biological activities. Structurally, chalcones are open-chain flavonoids having two aromatic rings joined by a three-carbon enone moiety. Chalcones comprises an important group of natural compounds that are especially abundant in fruits (e.g., citrus, apples), vegetables (e.g., tomatoes, shallots, bean sprouts, potatoes) and various plants and spices (e.g., licorice) (Di Carlo et al., 1999).

Based on this knowledge we proposed the use trans-chalcone as phenolic-rich functional foods providing potential nutritional benefits (Garcia-Alonso et al., 2003). The acceptance of bioflavonoids for health claims is also associated with the assessment of their toxic effects in

order a gain better insight for their approval. In fact, these polyphenolic compounds also known as dietary antioxidants also exhibit contrasting pharmacological actions, such as prooxidant activity and the induction of cell death (Lapidot et al., 2002; Saleem et al., 2002).

To perform physiological, biochemical and toxicological study by phytochemicals, the in vitro cell culture techniques has provided a added advantage (Glei et al., 2003). Our current research focuses on the validation of trans-chalcone for their cytoprotective activities against oxidant challenge in human liver HepG2 cell line. This cell line more closely resemble human hepatocyte in culture has been widely used in biochemical and nutritional studies (Ramos et al., 2005). Here we aimed to evaluate the modulatory effect of the trans-chalcone on cell proliferation and on the protection from oxidative stress caused by hydrogen peroxide, frequently used as models to study the mechanisms of cellular alteration resulting from free radical action.

Materials and Methods

Reagents and chemicals

Dulbecco's Modified essential medium (DMEM), penicillin, streptomycin, and L-glutamine were purchased from Gibco (Paisley, Scotland, UK). 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal

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bovine serum, trypsin, 1,10-phenanthroline monohydrate (o-phenanthroline), ethylenediaminetetraacetic acid (EDTA), N,N'-diphenyl-1,4-phenylene-diamine (DPPD), reduced glutathione (GSH), tert-butylhydroperoxide (t-BOOH) 70% solution in water and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, USA). Hydrogen peroxide (H₂O₂) aqueous solution 30% and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). All other reagents were of the highest grade obtainable.

Cell culture and treatments

HepG2 human caucasian hepatocyte carcinoma cells (National Centre for Cell Science (NCCS) Pune, Maharashtra, India) were routinely grown in DMEM supplemented with 10% (v/v) foetal bovine serum, 100 U/mL penicillin, 100 lg/mL streptomycin, in a humidified atmosphere of 95% air-5% CO₂ at 37°C. Stock solutions of H₂O₂ were freshly prepared in serum-free DMEM. At the treatment stage, the final DMSO concentration was never higher than 0.1%.

Proliferative effect of t-chalcone on HepG2 cells

The MTT-assay (Mosmann, 1983) was used to evaluate the antiproliferative activities of the juice extracts. The assay is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells, which can be photometrically quantified. An increase in the number of living cells results in an increase in total metabolic activity, which leads to a stronger color formation. For the assays, cells (5 x 10³ cells/well in 200 µL of complete DMEM) were placed in each well of a 96 well flat bottom plate. Cells were allowed to adhere for overnight, and then treated with 20 µM of trans-chalcone for 6 h. Blank wells contained the above concentrations of juice extracts in 200 ul of growth medium but with no cells. After completion of incubation period, 20 ul MTT (5mg/ml) was added to each well for 2h. Following which media was removed and 100 ul of DMSO were added to each well in order to solubilize the formazan. The plate was read using a ELISA reader at a wavelength of 540 nm. The results are expressed as the percentage of viable cells with respect to the control.

Lipid peroxidation assays

HepG2 cells (1x10⁵ cells) were seeded onto 60 mm Petri dishes and medium was replaced by 3 ml DMEM containing 20 µM t-chalcone concentration for 6 h. N,N'-diphenyl 1,4-phenylene-diamine (DPPD) o-phenanthroline were added to culture 5 min prior to the addition of H₂O₂ (20mM for 2 hr). After the treatment cells were washed twice in prewarmed PBS (pH-7.2) and scraped into 2ml PBS, cell suspension were used for protein determination and thiobarbituric acid reacting substances (TBARS) assay (Wright et al., 1981). After 1 h of incubation of cell suspension, 1.0 ml of 5% TCA and 1.0 ml of 0.67% TBA were added in samples. The reaction mixture from the vial was transferred to the tube and centrifuged at 3,500 rpm for 15 min. The supernatant was transferred to another tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were

cooled and the absorbance of the color was read at 535 nm. The rate of lipid peroxidation was expressed as nmol of malonaldehyde (MDA) formed /mg protein.

Enzyme activities

The activities of the glutathione related enzymes, glutathione peroxidase and glutathione s-transferase, which are involved in the reduction and detoxification process were also evaluated. For the experiment, 105 cells were seeded onto 60 mm cell culture plates. After overnight incubation, the media was removed and cells were exposed to 20µM trans-chalcone for 6 hr. Following the treatment the cells were washed twice in serum free medium and later challenged with 20mM H₂O₂ for 2 hr. Further cells were detached by trypsinisation (0.25% trypsin and 0.25 % EDTA in PBS), warmed twice in prewarmed PBS (pH 7.2) and resuspended in PBS. Cells were then disrupted by sonication and supernatant was used for protein determination.

Assay of glutathione peroxidase activity

Coupled enzyme assay with glutathione reductase (GR) is used for the estimation of GPx activity. The glutathione disulphide produced as a result of GPx activity, which is immediately reduced by GR thereby, maintaining a constant level of reduced glutathione in reaction system. The assay takes advantage of the concomitant oxidation of NADPH by GR, which is measured at 340 nm. Specific activity of the enzyme was measured according to the procedure described by Mohandas et al., 1984. The reaction mixture consisted of phosphate buffer (0.05 M, pH 7.0), 1 mM EDTA, 1mM sodium azide, 1mM glutathione, 0.2 mM NADPH, 0.25 mM hydrogen peroxide and cell suspension (10% w/v) in a final volume. The disappearance of NADPH was recorded at 340 nm at room temperature. The enzyme activity was calculated as nMole NADPH oxidized/min/mg protein by using molar extinction coefficient of 6.22 x10³ M⁻¹cm⁻¹.

Assay of glutathione-s-transferase activity

GST activity was determined according to the method of Habig et al. (1974). In this assay, GST catalyzes the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB), producing a dinitrophenyl thioether chromophore which is accompanied by the appearance of an absorption band at 340 nm. For the assay, the following solutions were added sequentially to a semi-micro quartz glass cuvette: CDNB working reagent, 20 mM reduced glutathione (GSH). The increase in absorbance was monitored at 340 nm at 25 oC over a 3-min time period.

Results

Proliferative effect of t-chalcone on HepG2 cells

In the present study we used 1, 3, diphenyl prop-2-ene-1-one (trans-chalcone) for its proliferative effect on hepatocellular carcinoma (HepG2) cells. Treatment of HepG2 cells with trans-chalcone resulted in a dose dependent inhibition of cell proliferation. It is evident from the figure that upto 40 µM concentrations there is

Effect of trans-chalcone on GSH related enzymes activities

The cellular defense systems (including glutathione, glutathione-related enzymes, and antioxidant and redox enzymes) play a crucial role in cell survival and growth in aerobic organisms. Exposure to 20 mM H₂O₂ for 2 h significantly increased the activity of both GPx and GST, compared with control cells. Preincubation of cells 20 μM for 6 h partially negated the H₂O₂ induced increase in GPx activity.

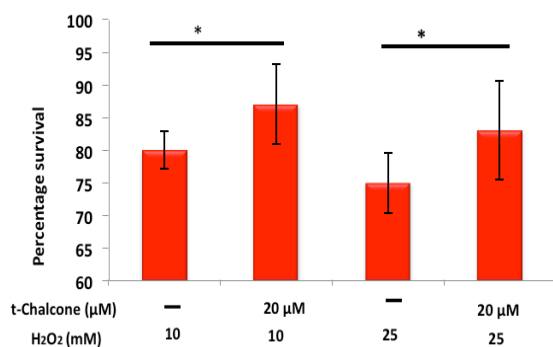


Figure 1. The Effect of Trans-chalcone on Oxidant-Induced Cytotoxicity in HepG2 Cells. **p*<0.05

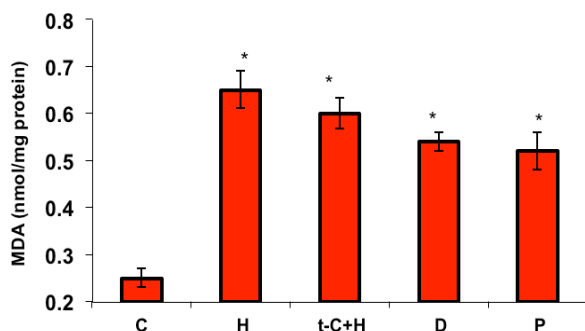


Figure 2. The Effect of the Trans-chalcone on Oxidant Induced Lipid Peroxidation in HepG2 Cells. **p*<0.05

no significant growth inhibition and viability of cells start decreasing as the concentration increased.

Protective effect of trans-chalcone in HepG2 cells against hydrogen peroxide induced cytotoxicity

As evident from fig 3.1, trans-chalcone concentration equal to or below 20 μM had no significant inhibitory effect on HepG2 cells growth. For this reason, this concentration was chosen for subsequent experiments aiming to evaluate the cytoprotective effects against the H₂O₂, widely used model of in vitro oxidative stress. Figure 1 shows the effect of preincubation of trans-chalcone on HepG2 cell death. Preincubation of cells with trans-chalcone at 20 μM concentrations for 6 h partially abolished the cell death induced by H₂O₂ with an increase in cell viability by 8.7 % and 10.6 % for 10 mM and 25 mM of H₂O₂.

Lipid Peroxidation

As a biomarker for lipid peroxidation, the cytoplasmic concentration of malonaldehyde (MDA) was measured in cells treated with H₂O₂ (20 mM 2 h) which had been pretreated for 6 h with 20 μM trans-chalcone. Fig. 3 showed the effect of preincubation with trans-chalcone on H₂O₂-induced lipid peroxidation. Exposure of cells to 20 mM H₂O₂ for 2 h significantly increased lipid peroxidation. As illustrated preincubation of cells with compound at 20 μM for 6 h prevented t-BOOH-induced lipid peroxidation. Importantly, as can also be seen the t-chalcone alone were not prooxidant and did not increase lipid peroxidation at the concentrations. The concentration of 20 μM was seen to be effective in preventing lipid peroxidation. The addition of intracellular iron chelator o-phenanthroline (25 μM) and the radical scavenger DPPD (10 μM) partially abolished H₂O₂ induced lipid peroxidation.

Discussion

In our study, t-chalcone concentration equal to or below 20 μM had no effect on HepG2 cell growth. For this reason, this concentration range was chosen for subsequent experiments aiming to evaluate the cytoprotective effects of this against oxidative challengers. For that, HepG2 cells were preincubated with juice extracts at different sub-toxic concentrations of t-chalcone compounds and challenged with hydrogen peroxide, widely used model of in vitro oxidative stress. It is well known that H₂O₂ can directly damage DNA, lipids, and other macromolecules, causing oxidative injury to the cell. Similarly, the organic hydrogen peroxide induces an array of cellular dysfunctions, including peroxidation of membrane lipids, glutathione and protein thiol depletion, alteration of calcium homeostasis, and DNA damage, eventually leading to cell death. In agreement with this, other previously reported data (Feng et al., 2002; Hong and Liu, 2004; Lazze` et al., 2003; Nardini et al., 1998; Sohn et al., 2005; Yau et al., 2002), the results of the present study demonstrated that cells preincubated with t-chalcone showed an increased resistance to oxidative challenge. This was revealed by an increase in cell survival and a decrease in the formation of lipid peroxidation products after exposure to H₂O₂. Lipid peroxidation has been suggested to play an important role in the development of toxicity. It is thought that peroxidation of the cell membrane phospholipids and an accumulation of lipid peroxides alter membrane fluidity and permeability, leading to disruption of membrane structure and function (Sohn et al., 2005). In the present study, the protective effect exerted by compound was observed after a short preincubation period (6h). Interestingly, the t-chalcone rapidly afforded protection from H₂O₂-induced lipid peroxidation, as revealed by the decrease in malondialdehyde production even after a preincubation period of 2h, indicating that t-chalcone was rapidly taken up by cells, whose antioxidant defenses were thus enhanced. In this context, it is widely believed that the antioxidant capacity of polyphenols mainly resides in their ability to scavenge the free radicals generated during lipid peroxidation (Arora et al., 1998). However, in addition to its radical scavenging activity, polyphenols possess an ideal structural chemistry for metal chelation, supporting the role of polyphenols as preventative antioxidants in terms of inhibiting transition-metal catalyzed free radical formation (Rice-Evans et al., 1997). In this regard, it has been shown that cell death induced by both H₂O₂ and t-BOOH depends on a cellular source of iron. Both peroxides react with ferrous iron to produce more potent oxidants, the hydroxyl and t-butyl alkoxy radicals,

respectively, by the Fenton reaction (Farber et al., 1990). Thus, iron chelation will decrease the toxicity of oxygen to cells and may contribute to the cytoprotective activity of phenolic compounds. Accordingly, dietary plant phenolics such as quercetin or various caffeic acid derivatives have been reported to protect from H₂O₂ and t-BOOH-induced toxicity by an iron-chelating mechanism (Sestili et al., 2002). Furthermore, anthocyanins (Lazze` et al., 2003) and caffeic acid (Nardini et al., 1998) protect cell systems from t-BOOH-induced toxicity and lipid peroxidation.

For comparison, in the present study, we also evaluated the ability of the radical scavenger DPPD and the intracellular iron chelator o-phenanthroline to prevent lipid peroxidation. Under our experimental conditions, both DPPD and o-phenanthroline prevented lipid peroxidation caused by either H₂O₂. These data indicate that both radical scavenging and iron chelation play a role in the prevention of the lipid peroxidation caused by H₂O₂. The cellular defense system (including glutathione, glutathione-related enzymes, and antioxidant and redox enzymes) plays a crucial role in cell survival and growth in aerobic organisms. Reduced glutathione (GSH), the major intracellular thiol, is believed to be an important protector against free radical damages by providing reducing equivalents for antioxidant enzymes and also by scavenging hydroxyl radicals and singlet oxygen (Lee et al., 2002).

Among the GSH-related enzymes, in the present study we measured the activity of glutathione peroxidase (GPx), and glutathione-s-transferase (GST). The enzyme GPx participates in the detoxification of t-BOOH by converting t-BOOH to t-butanol and oxidized glutathione (GSSG) (Sohn et al., 2005). When formed, GSSG is quickly recycled back to GSH by the enzyme glutathione reductase. The enzyme glutathione-s-transferase (GST) catalyzes the conjugation of reduced glutathione (GSH) to a wide variety of hydrophobic molecules, thereby reducing lipid hydroperoxides and providing protection from membrane lipid peroxidation. Increases in the activities of these GSH-related enzymes have been reported in cell systems after exposure to t-BOOH and concomitant with a decrease in reduced GSH levels (Aniya and Daido, 1994). In agreement with these findings, we observed a significant increase in both GPx and GST activity after exposure to t-BOOH. Preincubation with the 20µM t-chalcone effectively suppressed the increase in GPx activity, but not that of GST. Consistent with our results, Murakami et al (2002) reported the ability of tea catechins to suppress the t-BOOH-induced increase in GPx activity in HepG2 cells. Thus, as regards GPx activity, trans-chalcone in the juice might exert their protective effect by delaying the consumption of GSH and/or other cellular antioxidants.

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