Naringin Attenuates CCl4-Induced Hepatocyte Damage by Inhibiting Endoplasmic Reticulum Stress

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Abstract

Objective: Exposure of the liver to diverse chemicals induce hepatic damage. Carbon tetrachloride (CCl4) is widely used toxin to investigate hepatic injury. In our study, the effects of naringin (NRG), a flavanone abundant in citrus fruits, on endoplasmic reticulum (ER) stress and stress-mediated apoptosis in CCl4-induced liver injury were investigated. *Materials and methods:* THLE-3 cells were exposed to varying concentrations of CCl4 for 24 hours and then treated with different doses of NRG for 4 hours. The effects of varying concentrations on cell viability were determined. Then, protein expressions of ER stress markers were detected in hepatocytes. Finally, Bcl2 active / inactive cell ratios were determined by flow cytometry. *Results:* NRG treatment (5 and 10 µM) significantly increased cell viability, which decreased with CCl4 administration. Similarly, the increased levels of ER stress markers as a result of CCl4 application were significantly reduced with NRG treatment. Finally, NRG prevented apoptosis by significantly reducing ratio of Bcl2 inactive cells. *Conclusion:* NRG treatment is effective in suppressing ER stress in CCl4-induced hepatocyte damage and preventing ER stress-induced apoptosis.

Keywords

Liver damage

Naringin

Endoplasmic reticulum stress

Apoptosis

1. Introduction

The prevalence of chronic liver diseases has increased significantly in recent years in young and elderly populations that had increased exposure to chemical toxins, viruses and drugs [1]. Persistent and progressive liver disorders cause severe liver damage due to increased loss of cell and tissue function and are associated with high mortality [2]. The management of liver injury remains one of the most challenging issues in contemporary medicine [3] and there is a need to

develop new treatments for acute liver injury.

Carbon tetrachloride (CCl4) metabolism catalyzed by hepatic microsomal cytochrome P450 produces free radicals that rapidly deplete hepatic glutathione and induce a chain reaction of lipid peroxidation of the hepatocyte membrane [4]. This CCl4 metabolism in the endoplasmic reticulum (ER) leads to an unfolded protein response as an indicator of loss of ER function. This defect triggers ER stress, which has been implicated in many liver diseases. The unfolded protein response has been observed to occur in fatty liver disease, obesity, hepatic insulin resistance, ischemia-reperfusion injury, hepatitis B/C, alcohol-induced liver injury and many toxic agent-induced diseases including acetaminophen toxicity [5]. Therefore, CCl4 is widely used in experimental models of hepatotoxicity induced by xenobiotics [6].

The unfolded protein response is sensed by the binding immunoglobulin protein (BIP, GRP78) and activated upon accumulation of unfolded proteins in the ER. The accumulation of unfolded proteins leads to the release of BIP and stimulation of three ER transmembrane proteins. These proteins are inositol-requiring enzyme 1 (IRE), pancreatic ER kinase (PERK) and activating transcription factor 6 (ATF6). PERK phosphorylates eukaryotic initiation factor (EIF2α), leading to attenuation of overall translation in the cell. It also increases the expression of several mRNAs, including the ATF4 transcription factor and its target CCAAT-enhancer-binding homologous protein (CHOP). IRE1 autophosphorylation drives the production of the active transcription factor truncated X-box binding protein 1 (sXBP1). ATF6 is truncated in the Golgi to its active form and transferred to the nucleus. These are all adaptations to resolve protein folding defects and increase cell survival. If the stress becomes constant, this adaptation will fail, leading to impaired ER function. At the end of all these, the pro-apoptotic transcription factor CHOP, which is the target of the unfolded protein response, is activated and apoptosis is triggered by the formation of reactive oxygen species [5,7,8].

Naringin (NRG) is a natural flavonoid glycoside obtained from grapefruit and orange [9]. It belongs to the flavonoid group with a fifteen-carbon structure and has been widely shown to have anti-inflammatory/antioxidant effects [10-11]. It has been reported that flavonoids included in this group may be potential therapeutics for the prevention or treatment of cancer and chronic diseases [12,13]. In addition, anti-inflammatory, antiapoptotic and antioxidant properties have been shown against CCl4 toxicity in mouse hepatocytes [14]. However, the effects of NRG on CCl4-induced ER stress in hepatocytes have not been clarified yet. In this study, the effects of NRG on normal human hepatocyte toxicity were shown through ER stress markers.

2. Methods

2.2. Cell culture and chemicals

The normal human hepatocyte cell line THLE-3 was purchased from American Type Culture Company (ATCC, MD, USA) and maintained according to the supplier's instructions. Cells were cultured in BEGM (Lonza/Clonetics Corporation, Walkersville, MD 21793 Cat. No: CC3170) medium in a 37° C and 5% CO_2 incubator throughout the study. NRG (Sigma-Aldrich, Product Code: 71162) used in the experiments was dissolved in DMSO and prepared at various concentrations for toxicity experiments. DMSO concentrations were kept at a concentration of lower than 0.5% so that there would be no effect on cell viability. CCl4 was obtained from Sigma-Aldrich (Product Code: 289116) and prepared fresh for each experiment.

2.3. Determination of cell viability

CCl4 (Sigma-Aldrich; Product Code: 289116) at concentrations of 1, 5, 10 and 20 mM was applied for 24 hours to induce acute damage to the cells in 90-six well plates [6,15] and the appropriate CCl4 concentration

was determined. Cells were treated with different concentrations of NRG (1, 5 and 10 μM) [16] for 4 hours and then treated with CCl4 for 24 hours. The effects of NRG on cell viability were evaluated by methylthiazole diphenyl tetrazolium (MTT) assay. After the treatments, 5 μl of MTT solution was transferred to the wells and incubated for 1.5 hours. The medium in the plates was discarded and 200 μl DMSO was added to each well. Absorbances were measured at 570 nm with a spectrophotometer.

2.4. Determination of ER stress markers

For the preparation of cell lysates, cells in six-well plates were diluted with PBS (pH 7.4) to 1 million cells per ml. Cells were subjected to repeated freeze-thawing. Then centrifuged at 3000 rpm for 20 minutes and supernatant was taken. Total protein amounts in the groups were determined by Sigma, Total Protein Kit (Product code: TP0100). ER stress markers CHOP (Cat. No: MBS7720630), GRP78 (Cat. No: MBS3801061), ATF6 (Cat. No: MBS018412), ATF4 (Cat. No: MBS726369), IRE1 (Cat. No: MBS7720641), and PERK (Cat. No: MBS1608105) were determined according to MyBioSource, Inc. ELISA kit protocol.

2.5. Determination of Bcl-2 Activation

Bcl-2 phosphorylation (Ser70) and total Bcl-2 expression levels in the cells were detected with "Muse™ Bcl-2 Activation Dual Detection Kit (Cat. No: MCH200105, Luminex)" using Muse cell analyzer according to the manufacturer's protocol. As a result of the test, Bcl-2 active cell ratio (by phosphorylation), inactive cell ratio and Bcl-2 non-expressing cell ratios in the groups were determined.

2.6. Statistical analyses

Data were analyzed using GraphPad Prism version 8.0 software. The values of the data were presented as mean ± SD. The variables measured in each group were analyzed by Shapiro-Wilk test to determine their conformity to normal distribution. Since all data showed normal distribution, the data of the variables were evaluated by One-Way Analysis of Variance (ANOVA). In variables with significant differences, pairwise comparisons between groups were corrected by Bonferroni test. The results determined as $p < 0.05$ were considered significant.

3. Findings

3.1. Effects of CCl4 and naringin on cell viability

In order to determine the effects of CCl4 on cell viability, THLE-3 cells were treated with 1, 5, 10 and 20 mM CCl4 for 24 hours. Our results showed that cell viability decreased in a dose-dependent manner. Significant differences were found in the 5 mM treated group ($p = 0.006$), 10 mM treated group ($p < 0.001$) and 20 mM CCl4 treated group ($p < 0.001$) compared to the control group (**Figure 1a**). 10 mM, at which more than 50% of the cells remained viable, was determined as the CCl4 concentration to be used in the experiment.

In order to determine the effects of NRG on cell viability, 3 different concentrations of NRG (1, 5 and 10 μM) were applied to the cells for 4 hours after 10 mM CCl4 treatment for 24 hours (**Figure 1b**). Our results showed that the viability of cells treated with 5 μM ($p = 0.013$) and 10 μM ($p = 0.005$) NRG was significantly increased compared to the 10 mM CCl4 control group. Further experiments were conducted with 10 μM, the highest NRG concentration with statistical significance.

3.2. Effects of naringin on ER stress markers

To induce ER stress in THLE-3 cells, cells were treated with 10 mM CCl4 for 24 hours (6, 15). Then the cells were incubated with 10 μM NRG for 4 hours. Our results showed that ER stress marker levels of GRP78, ATF6, ATF4, IRE1, PERK and CHOP were
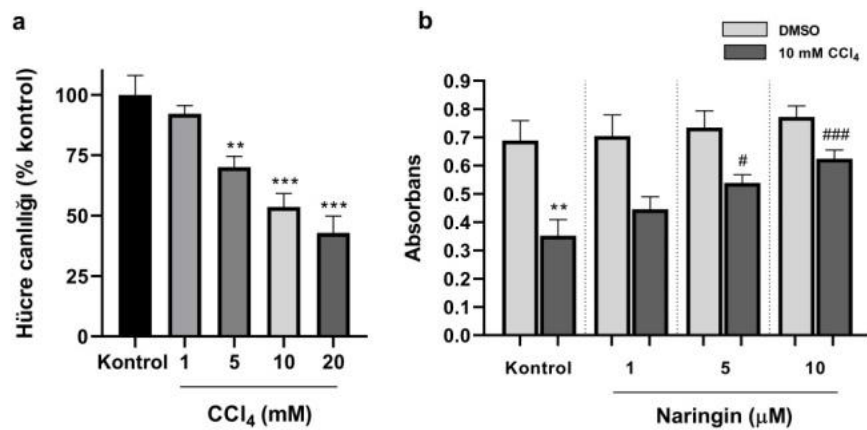
Figure 1. Effect of naringin on CCl4-induced cell death. **(a)** THLE-3 cells were treated with 1, 5, 10 and 20 mM CCl4 for 24 hours and cell viability was evaluated by MTT assay. **(b)** THLE-3 cells were treated with 10 mM CCl4 for 24 hours and then treated with 1, 5 and 10 μM NRG for 4 hours. DMSO was applied to the control group. Cell viability was evaluated by MTT assay. The results reflect three independent experiments. Data are presented as mean ± SD. **p < 0.01, ***p < 0.001 indicates significant differences compared to the control group and #p < 0.05, ####p < 0.001 indicates significant differences compared to the 10 mM CCl4 group. CCl4, carbon tetrachloride; MTT, methylthiazole diphenyl tetrazolium; NRG, naringin.
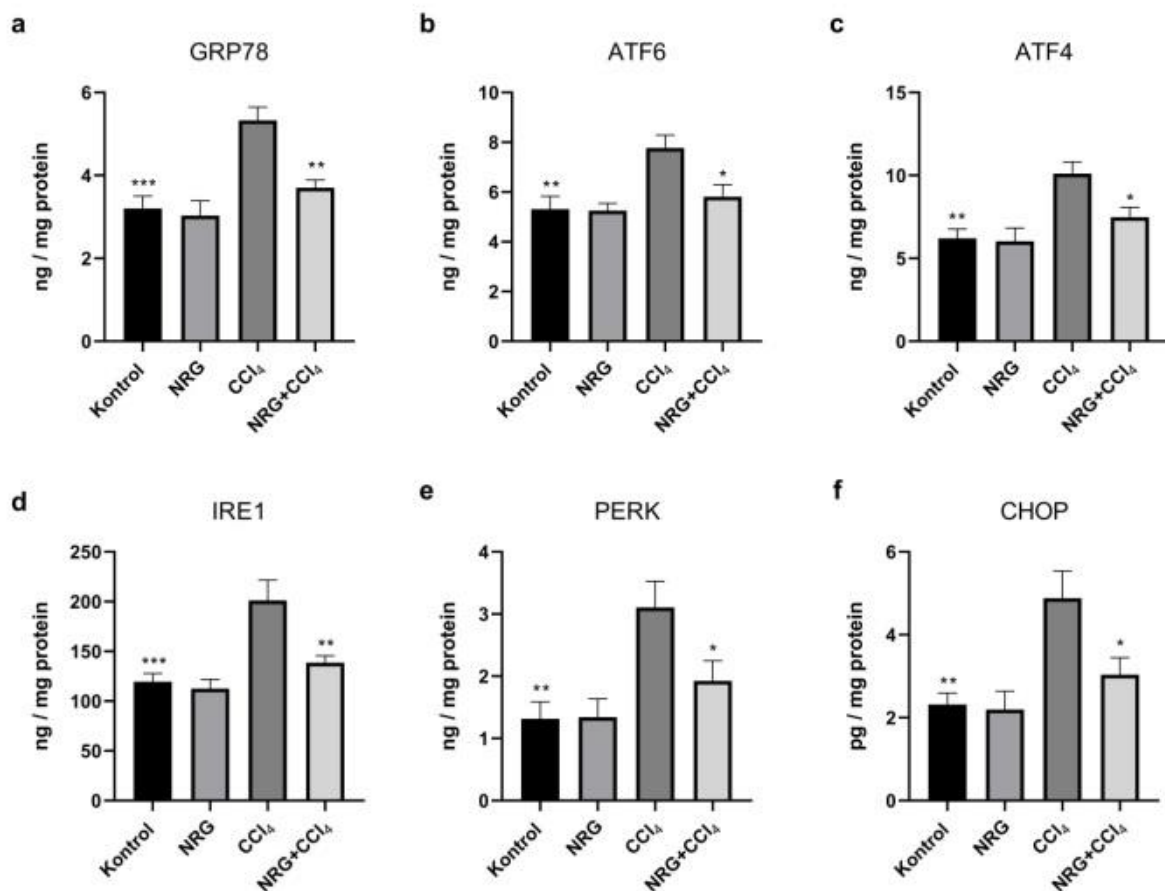

Figure 2. Effect of naringin on CCl4-induced ER stress. **(a–f)** THLE-3 cells were treated with 10 mM CCl4 for 24 hours and then cells were treated with 10 μM NRG for 4 hours. Protein levels of ER stress markers were evaluated by ELISA. Results reflect three independent experiments with three replicates. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 indicates significant differences compared to CCl4 group. CCl4, carbon tetrachloride; NRG, naringin; GRP78, binding immunoglobulin protein; IRE1, inositol-requiring enzyme 1; PERK, pancreatic ER kinase; ATF6, activating transcription factor 6; ATF4, activating transcription factor; CHOP, CCAAT-enhancer-binding homologous protein.

significantly increased in the CCl4 group compared to the control group. In the NRG-treated group, a significant decrease was detected in all protein levels compared to the CCl4 group (**Figure 2a–f**). On the other hand, no statistically significant difference was detected between the control group and NRG group.

3.3. Effects of naringin on Bcl-2 phosphorylation

In order to determine the apoptosis induced by CCl4-induced ER stress in cells, Bcl-2 active/inactive cell ratios were determined by flow cytometry method. As shown in **Figure 3**, CCl4 treatment significantly increased the anti-apoptotic Bcl-2 inactive cell ratio ($p < 0.001$). NRG treatment resulted in a significant decrease in the Bcl-2 inactive cell ratio increased by CCl4 ($p < 0.001$). No difference was detected between the control group and NRG group.

4. Discussion

Liver diseases are considered a significant problem worldwide. Many environmental toxins cause liver damage in humans [17]. Despite the new developments in hepatology, the treatment methods used for liver diseases remain inadequate in addressing the problems caused by such toxins. Eliminating the toxicity occurring at the cellular level of the liver, which is the organ that is most affected by toxic substances, will be beneficial in the treatment of liver diseases [18-19]. This study aims to investigate the effect of naringin, an active ingredient abundant in citrus fruits, on CCl4-induced ER stress and apoptosis in hepatocytes.

Bioactivation of CCl4 molecule to the trichloromethyl free radical CCl3 is carried out by cytochrome P450 isozymes (P450s) in hepatocytes. Among the various forms of P450s present in hepatocyte ERs, P450 2E1 (CYP2E1) has been shown
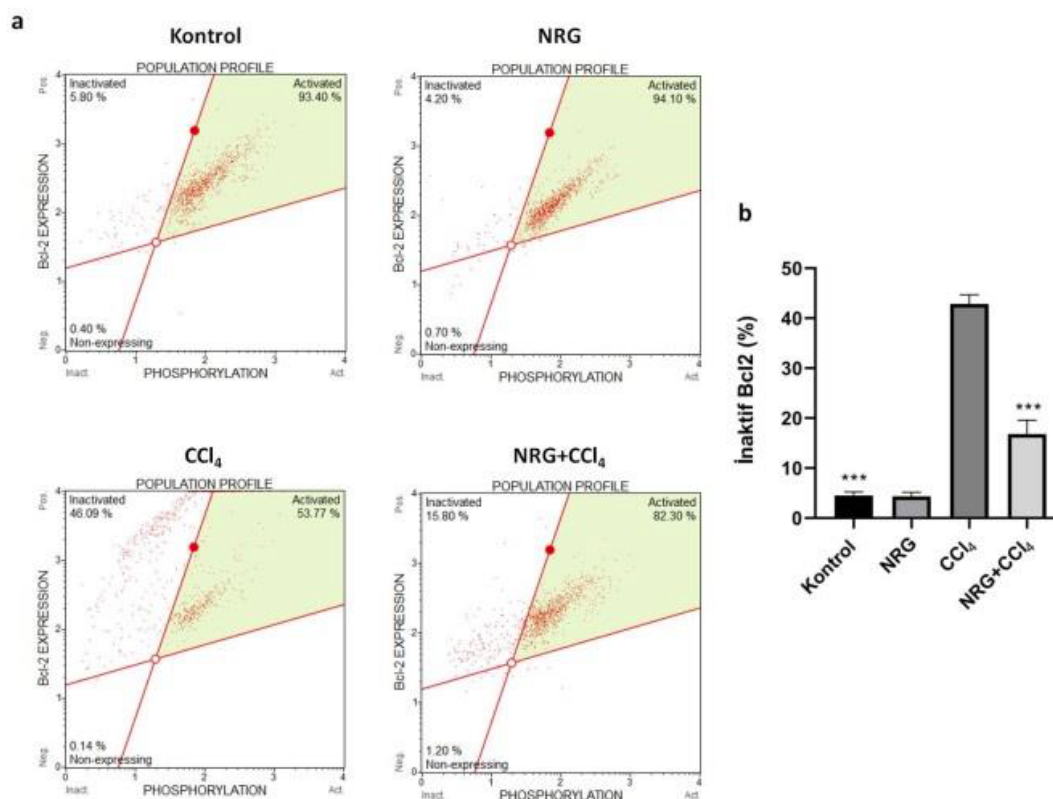

Figure 3. Effect of naringin on CCl4-induced Bcl-2 phosphorylation. **(a)** THLE-3 cells were treated with 10 mM CCl4 for 24 hours and then cells were treated with 10 μM NRG for 4 hours. **(b)** Bar graph shows the proportion of Bcl-2 inactive cells. Bcl-2 non-expressing, Bcl-2 active and inactive cell profiles in the cell population were determined by flow cytometry. **(b)** Bar graph shows the proportion of Bcl-2 inactive cells (%). Results reflect three independent experiments. Data are presented as mean ± SD. ***$p < 0.001$ indicates significant differences compared to CCl4 group. CCl4, carbon tetrachloride; NRG, naringin.

to be an important enzyme for CCl4 bioactivation and CCl4-mediated pathological changes. Activation of P450 requires electron transfer between NADPH-P450 reductase (NPR) and P450. During electron transfer, some electrons leak from the transfer system. This "electron leak" also significantly contributes to the generation of free radicals during the redox cycle. Several studies have demonstrated that these free radicals cause ER stress [20-22].

In response to liver damage, inflammation and cell death pathways stimulate hepatocytes for liver regeneration. However, in cases of persistent and severe toxicity, hepatic stellate cells undergo myofibroblast-like activation and synthesize more extracellular matrix proteins [23-24]. This will increase the need for protein synthesis, this will increase the protein folding process, which will overwork the ER. Three ER-resident transmembrane proteins, IRE1, PERK and ATF6, have been identified as ER stress sensors. The ER stress response reduces the translation of specifically secreted proteins through PERK activation and subsequent EIF2α phosphorylation to alleviate protein accumulation in the ER. Furthermore, cells under ER stress increase the protein folding capacity of the ER by up-regulating ER chaperones [25]. CCl4 has been shown to induce swelling of the smooth endoplasmic reticulum in hepatocytes, which leads to increased expression of GRP78 and sXBP1, a phenomenon associated with ER stress [26]. In our previous studies, we reported that CCl4 toxicity caused ER stress in rats [19,27]. In a study with HepG2 cells, CCl4-induced apoptosis and the response of ER stress to resveratrol treatment were investigated. The results showed a significant increase in the mRNA expressions of ER stress markers XBP-1, CHOP, IRE1, ATF6, ATF4, EIF2α, PERK and protein expressions of GRP78, IRE1 and PERK in CCl4-treated cells and a significant decrease in the expressions of key proteins in resveratrol-treated cells. Similarly, in this study, CCl4 treatment showed a significant increase in protein expression of ER stress markers in THLE-3 hepatocytes, in accordance with the literature.

Due to the therapeutic potential of their natural products, plants play an important role in the fight against various diseases. In order to maximize the therapeutic properties of these herbal medicines, it is necessary to improve the systems of obtaining them from natural sources [28]. Many compounds with antioxidant potential have been tested for their protective activity against CCl4 toxicity [29]. Previous studies have shown that naringin treatment can significantly increase the levels of non-enzymatic or enzymatic antioxidants (such as superoxide dismutase and catalase), inhibit the overexpression of CYP2E1, and suppress ROS formation leading to a rapid improvement in CCl4-induced liver damage. Transmission electron microscopy and histopathological analysis also showed that naringin effectively prevented CCL4-induced nucleus and mitochondria defects in hepatocytes. In addition, naringin significantly decreased the release of cytochrome c from mitochondria to cytosol and increased the expression of antiapoptotic proteins (Bcl-2 and Bcl-xL) [14]. The findings obtained from the research on the possible role of naringin in nuclear respiratory factor 2 (Nrf2) activation showed that naringin had cell protective effects through Nrf2-transduction and p38 inactivation [30]. For the first time in the literature, this study shows that naringin significantly decreased CCl4-induced ER stress markers in hepatocytes. Both the activation of inflammatory pathways and the stimulation of antioxidant defense systems in the cell during naringin treatment have proven effective in overcoming ER stress in hepatocytes.

It has been determined in many studies that CHOP plays a role in triggering ER stress-mediated apoptosis [31,32]. CHOP downregulates Bcl2 expression and upregulates BIM expression, leading to increased expression of proapoptotic proteins [33]. Our findings indicate that CCl4-induced ER stress in hepatocytes reduces the phosphorylation of antiapoptotic Bcl2, thereby limiting its activation. Naringin treatment showed a significant decrease in the inactive Bcl2 ratio,

indicating its potential in countering the effects of ER stress-mediated apoptosis.

Conclusion

The liver plays a vital role in regulating various metabolisms in the body such as fat, protein and carbohydrate metabolism, while also serving as a crucial site for the detoxification of foreign substances entering the body. The unfolded protein response is active in many acute and chronic liver diseases. Eliminating these folding defects and damages in the cellular mechanisms that cause these defects (such as free radical accumulation, inflammatory pathways) is critical for effective treatment. The results obtained from this research are significant in terms of potentially using new natural compounds for treatment and contributing to future studies. In conclusion, our study demonstrates that naringin can significantly alleviate ER stress induced by hepatocyte toxicity.

Disclosure statement

The author declares no conflict of interest.

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