Antioxidative activity of Chicoric acid through Nrf2/MAPK mediated HO-1 induction in RAW 264.7 cells

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Abstract

The antioxidative potential of chicoric acid was investigated through heme oxygenase (HO)-1, one of phase II enzymes and exhibits antioxidative activity, induction in RAW 264.7 cells. There were also measured intracellular radical scavenging activity and glutathione (GSH) concentration in chicoric acid treated RAW 264.7 cells. Chicoric acid treatment potently induced HO-1 expression, scavenged reactive oxygen species (ROS) and increased GSH concentration. Transcription factor of phase II enzymes, nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), was investigated and identified in accordance with HO-1 expression. In addition, chicoric acid treatment phosphorylated PI3K/Akt with slight activation of the extracellular regulated kinase (ERK). Chicoric acid-induced antioxidative potential protected RAW 264.7 cells against oxidative stress-induced cell death. In conclusion, chicoric acid potently accelerated Nrf2-mediated antioxidative potential through the modulation of PI3K/Akt pathway, which would contribute to their promising strategy for a novel anti-oxidative agent.

Keywords : antioxidative, HO-1, chicoric acid, Nrf2, PI3K/Akt

1. Introduction

Overproduced reactive oxygen species (ROS) initiated from oxidative stress damage lipids, proteins and DNA, which is implicated in the pathogenesis of several human diseases including atherosclerosis, cancer, neurodegenerative diseases and aging[1]. Therefore, many natural resources scavenging free radicals have been identified and they are proposed as therapeutic agents to counteract liver damage[2]. Among plant derived flavonoids, chicoric acid, a derivative of caffeic acid, showed potent antioxidative activity, but had only a weak anti-inflammatory effect [3,4].

Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes heme to biliverdin, carbon monoxide (CO), and free iron. The byproducts of HO-1 catabolism, CO and biliverdin/bilirubin, have been shown to exhibit protective effects against oxidative and inflammatory stimuli [5].
Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), a transcription factor for phase II enzyme induction, interacts with the antioxidant response element (ARE) to stimulate the expression of HO-1[6]. Mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)/Akt have been reported to regulate Nrf2 and are involved in extracellular signal-regulated kinase (ERK)-, c-Jun NH2-terminal kinase (JNK)-, p38-, and phosphoinositide 3-kinase (PI3K)/Akt-dependent cell survival and proliferation[7]. Upon stimulation with oxidative stress, Nrf2 induces a cellular rescue pathway that ameliorates the inordinate ROS production[8]. Therefore, nutraceutical agent that modulate Nrf2-mediated signaling cascades in activated macrophages might be a useful strategy for the treatment of excessive oxidative stress-issued diseases.

In this study, we explored the molecular mechanisms of chicoric acid, focusing on their role in the Nrf2-mediated oxidative signaling cascades in RAW 264.7 cells.

2. Materials and Methods

2.1. Reagents

Dulbecco’s modified Eagle Medium (DMEM), fetal bovine serum (FBS), and glutamine were obtained from Hyclone (Gaithersburg, MD, USA). Antibodies were purchased from Cell Signaling Technology (Boston, MA, USA) for phospho-ERK, ERK, phospho-c-JNK, JNK, phospho-p38, p38 and actin as well as Abcam (Cambridge, UK) for Nrf2 and HO-1, respectively.

2.2. Cell culture

The RAW 264.7 cell line was obtained from the American Type Culture Collection (TIB-71; Rockville, MD, USA) and cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. To investigate whether chicoric acid treatment induced the expression of HO-1 and its upstream signaling molecules, cells were treated with various doses and exposure time. In order to identify cytoprotective activity, oxidative stress was induced as previously described[9].
2.3. Cell viability assay

The 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 OD490.

2.4. Glutathione (GSH) content

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

2.5. ROS formation assay

ROS scavenging activity was measured by the cell permeable fluorescent dye DCFH-DA. RAW 264.7 cells were stained with 50 mM 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 to induce 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.

2.6. Western blot analysis

Total cell lysate was obtained from protein extraction solution (M-PER, Pierce biotechnology, Rockford, IL, USA). Protein samples (50 μg) from each lysate were separated on a 10% SDS-polyacrylamide gel and electrotransferred to PVDF membrane. Membrane was blocked for 2 hr at room temperature with 5% nonfat dry milk in TBST solution. The reactions were then incubated at 4°C overnight with primary antibodies in blocking buffer. After the membranes were washed, they were further incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody for 2 hr at room temperature. The blot was developed with enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology).

2.7. Statistical analysis

All data are expressed as mean ± SD. Statistical analyses were performed using SPSS version
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13.0 (SPSS Institute, Chicago, IL, USA). One-way ANOVA with Duncan’s multiple range tests was used to examine differences between groups. P values < 0.05 were considered significant, unless stated otherwise.

3. Results and Discussion

This study attempted to verify the antioxidative activity of chicoric acid through the evaluation of cytoprotective effect against t-BHP induced oxidative damage and further investigate the molecular mechanism on Nrf2-mediated HO-1 expression in RAW 264.7 cells. Oxidative damage caused sharp increase of ROS generation accompanied by accelerated cytotoxicity, which was significantly (p<0.05) attenuated by chicoric acid treatment in a dose dependent manner (Fig. 1). These results suggest that chicoric acid can role for a potent ROS scavenger against oxidative damage.

GSH is one of the primary defense systems against oxidative stress and roles as an essential intracellular reducing agent for maintenance of thiol groups on intracellular proteins and for antioxidant molecules. Fig. 2 indicates that chicoric acid treatment significantly (p<0.05) fortified intracellular GSH concentration in a dose dependent manner, which suggests that scavenging capacity of t-BHP-induced radicals was elevated in RAW 264.7 cells.

![Graph](image)

[Fig. 1] Chicoric acid scavenged t-BHP-induced ROS generation in RAW 264.7 cells.

The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at p<0.05 by Duncan’s multiple range test.
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HO-1 gene expression is stimulated by a variety of factors, including heme, hyperoxia, ROS, and naturally occurring antioxidants in various cell types[10]. Numerous studies have attempted to elucidate the function of HO-1 when its expression is upregulated as a cellular defense mechanism in response to various dietary phytochemicals[11]. Macrophages recognize and eliminate microbial pathogens, and the survival of macrophages might directly contribute to the host defense system. Several studies have shown that the virulence of some bacteria results from their ability to trigger the death of activated macrophages through stimulating ROS production[12]. Therefore, it is important to investigate these protective mechanisms and develop agents that are able to protect macrophages from ROS. As shown in Fig. 3A, chioric acid markedly increased HO-1 protein levels without inducing cytotoxicity (data not shown) in accordance with Nrf2 activation (Fig. 3B). Nrf2 is known to play an important role in the ARE-responsive expression of phase II enzymes, including HO-1[10]. In the oxidative stress response, the phosphorylated signaling kinases activate Nrf2, initiating the antioxidative and cytoprotective cascades[13].
Antioxidative activity of Chicoric acid through Nrf2/MAPK mediated HO-1 induction in RAW 264.7 cells

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[Fig. 3] Chicoric acid induced HO-1 expression in accordance with Nrf2 activation in RAW 264.7 cells.

(A) RAW 264.7 cells were treated for 12 hr with chicoric acid at the indicated concentrations.
(B) Chicoric acid induced Nrf2 activation in RAW 264.7 cells. RAW 264.7 cells were treated with indicated concentrations for 12 hr. Actin was used as an internal control.

MAPK and PI3K/Akt pathways are activated by the extracellular oxidative response, a signaling cascade that is facilitated by the Nrf2 transcription factor[14]. Therefore, Nrf2 is a pivotal transcriptional regulator for the induction of antioxidative genes, and MAPK and PI3K/Akt are key factors that induce Nrf2-mediated signaling transduction[15,8]. Chicoric acid dose-dependently induced PI3K/Akt phosphorylation but did not affect the phosphorylation of ERK, JNK, or p38 (Fig. 4).

[Fig. 4] Chicoric acid induced HO-1 expression depended on PI3K/Akt signaling pathway in RAW 264.7 cells.

Chicoric acid induced phosphorylation of Akt and partly ERK in RAW 264.7 cells, respectively. Actin was used as an internal control.
[Fig. 5] The antioxidative potential of chicoric acid against the t-BHP-induced oxidative damage in RAW 264.7 cells

The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at p<0.05 by Duncan’s multiple range test.

To confirm whether PI3K/Akt activation was required for HO-1 protein expression, we examined whether this signaling molecule is involved in the HO-1-mediated cytoprotective effect against t-BHP-induced oxidative stress. As shown in Fig. 5, chicoric acid-induced HO-1 upregulation protected macrophages from oxidative stress-induced cell death. In addition, the cytoprotective mechanism was confirmed by the PI3K/Akt selective inhibitor, LY294002.

In conclusion, these results suggest that chicoric acid strengthens antioxidative potential through GSH restoration and HO-1 expression. Moreover, chicoric acid issued HO-1 upregulation was mediated by Nrf2 and PI3K/Akt signaling pathway in RAW 264.7 cells. Therefore, chicoric acid, showed potent antioxidative properties, might be proposed as a potential therapeutic agent against oxidative stress.

References


