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Iron depletion in HCT116 cells diminishes the upregulatory effect of phenethyl isothiocyanate on heme oxygenase-1

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ABSTRACT

Some of the health-promoting properties of cruciferous vegetables are thought to be partly attributed to isothiocyanates. These phytochemicals can upregulate the expression of certain cytoprotective stress genes, but it is unknown if a particular nutrient is involved. Herein, the objective was to ascertain if adequate iron is needed for enabling HCT116 cells to optimally express heme oxygenase-1 (HO-1) when induced by phenethyl isothiocyanate (PEITC). PEITC increased HO-1 expression and also nuclear translocation of Nrf2, which is a transcription factor known to activate the HO-1 gene. However, in HCT116 cells that were made iron-deficient by depleting intracellular iron with deferoxamine (DFO), PEITC was less able to increase HO-1 expression and nuclear translocation of Nrf2. These suppressive effects of DFO were overcome by replenishing the iron-deficient cells with the missing iron. To elucidate these findings, it was found that PEITC-induced HO-1 upregulation can be inhibited with thiol antioxidants (glutathione and *N*-acetylcysteine). Furthermore, NADPH oxidase inhibitors (diphenyleneiodonium and apocynin) and a superoxide scavenger (Tiron) each inhibited PEITC-induced HO-1 upregulation. In doing so, diphenyleneiodonium was the most potent and also inhibited nuclear translocation of redox-sensitive Nrf2. Collectively, the results imply that the HO-1 upregulation by PEITC involves an iron-dependent, oxidant signaling pathway. Therefore, it is concluded that ample iron is required to enable PEITC to fully upregulate HO-1 expression in HCT116 cells. As such, it is conceivable that iron-deficient individuals may not reap the full health benefits of eating PEITC-containing cruciferous vegetables that via HO-1 may help protect against multiple chronic diseases.

1. Introduction

Certain edible plants contain natural substances that are believed to be important dietary components for helping to maintain good health. In particular, so-called cruciferous vegetables (e.g., watercress and broccoli) are abundant in phytochemicals known as glucosinolates, which are the precursors to the class of bioactive products known as isothiocyanates (Fahey et al., 2001). Two specific examples of widely-studied isothiocyanates are phenethyl isothiocyanate (PEITC) and sulforaphane. These isothiocyanates along with other related compounds are viewed as hormetic phytochemicals (Son et al., 2008), and conceptually, they can have both desirable and undesirable effects. Nevertheless, a recent compilation of research (Fimognari et al., 2012) suggests that, despite their genotoxic potential, natural isothiocyanates in proper dietary amounts are chemopreventive agents against numerous chronic diseases often linked to persistent oxidative stress and inflammation.

One possible mechanism of glucosinolates, or isothiocyanates, is that they increase the expression of numerous cytoprotective genes (Abdull Razis et al., 2010; Saw et al., 2011). Among this inducible gene cluster is heme oxygenase-1 (HO-1), whose upregulated expression by several isothiocyanates has been studied in detail (Prawan et al., 2008). HO-1 is important because it apparently plays a role in preventing inflammation and associated oxidative stress, as supported by the results of genetic studies dealing with human HO-1 deficiency (Yachie et al., 1999; Kawashima et al., 2002). Because HO-1 gene expression is increased by isothiocyanates (Prawan et al., 2008), the anti-inflammatory effects of isothiocyanates (Dey et al., 2006; Cheung et al., 2009) are thought to be attributed largely to HO-1.

A potential relationship between phytochemicals and micronutrients is unclear. After they are ingested and absorbed, micronutrients and phytochemicals can eventually become present together in cells. The former substances are essential, and the latter substances are non-essential, for the subsistence of cells. Isothiocyanates can upregulate the expression of several cytoprotective stress genes including HO-1 (Abdull Razis et al., 2010; Saw et al., 2011; Prawan et al., 2008), at least in cells that are adequately provided with all of the proper nutrients. However, it is unknown if such gene upregulation can occur when cells are deficient in a particular nutrient. Nevertheless, it is possible that micronutrients and phytochemicals could interact to help sustain and safeguard cells for good health. Because of this possibility, the novel question is raised from a health perspective whether

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individuals that are deficient in a single micronutrient can benefit optimally from certain phytochemicals that are believed to inhibit oxidative stress and inflammation associated with the development of multiple chronic diseases. To initiate basic research along this line, the objective of this study was to examine whether a specific micronutrient can influence the ability of a specific phytochemical to influence cytoprotective gene expression. We tested the hypothesis that adequate availability of the micronutrient, iron, is required to enable the phytochemical, PEITC, to fully upregulate HO-1 expression in HCT116 cells.

2. Materials and methods

2.1. Materials

HCT116 human colon adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). All fine chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), and general reagents were obtained from Fisher Scientific Co. (Norcross, GA), unless otherwise stated. Goat HO-1 polyclonal antibody (C-20), rabbit Nrf2 polyclonal antibody (H-300), rabbit anti-goat IgG-HRP, goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse β-actin monoclonal antibody (AC-15) was obtained from Sigma Chemical Co. (St. Louis, MO). The goat anti-rabbit IgG conjugated to Alexa Fluor 488 was obtained from Invitrogen (Carlsbad, CA).

2.2. Cell culture and treatment

HCT116 cells were cultured in McCoy's 5 A medium containing 10% fetal bovine serum and antibiotic-antimycotic agents (penicillin, streptomycin, and amphotericin B) until the cells reached a confluency level of 70–80% before experimentation. Preliminary work was performed to empirically determine suitable experimental conditions (i.e., reagent concentrations and incubation times) for best observing any treatment effects. The cells were made iron-deficient by pretreating them with 10–100 μM of either desferrioxamine (DFO) or 2′,2′-dipyridyl (DPD) for 16–24 h, before incubating these iron-deficient cells with 0–10 μM PEITC (dissolved in absolute ethanol with a final solvent concentration of 0.1%) for 2–5 h depending on the particular experiment. In "iron rescue" experiments, the iron-deficient cells were treated with 50–200 μM ferrous sulfate for either 4 or 8 h, before incubation with PEITC. In other experiments, HCT116 cells were pretreated with 10 mM of either *N*-acetylcysteine or glutathione for 2 h, and also were pre-treated with 2–20 μM diphenyleneiodonium (DPI), 1–20 mM Tiron, 0.1–1 mM apocynin, or 1–2 μM rotenone for 0.5–1 h, before incubating these cells with 10 μM PEITC.

2.3. Determination of HO-1 mRNA expression

Using an RNeasy Mini kit (Qiagen Inc., Valencia, CA), total RNA was isolated from the cells. HO-1 mRNA expression was evaluated by multiplex relative RT-PCR analysis of the total RNA. A Qiagen OneStep RT-PCR kit and gene-specific primers were utilized. The primer sequences for HO-1 were: forward, TGTGGCAGCTGTCTCAAACCTCCA; reverse, TTGA GGCTGAGCCAGGAACAGAGT. To normalize the data with an internal standard, a β-actin/competimer pair (Ambion Inc., Austin, TX) was employed. The RT–PCR conditions were 30 min at 50 °C followed by 15 min at 95 °C (RT), then 0.5 min at 95 °C, 0.5 min at 60 °C, and 1 min at 72 °C (PCR) for 25 cycles. The two cDNA products corresponding to HO-1 (175 bp) and β-actin (294 bp), were separated by 2% agarose gel electrophoresis with ethidium bromide staining. Densitometry of the separated gel bands was performed with a Kodak Image Station 440CF system to in turn generate HO-1 to β-actin ratios.

2.4. Determination of HO-1 and Nrf2 protein expression

After harvesting cells by scraping and centrifugation (500*g* for 5 min), they were washed with phosphate buffered saline (PBS) and recentrifuged. The cell pellets were sonicated in lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and Roche Complete Protease Inhibitor Cocktail, pH 7.4) and placed in ice for 30 min. The lysed samples were centrifuged at 16,000*g* for 20 min at 4 °C. The protein concentration of the collected supernatant was determined by the BCA protein assay (Pierce, Rockford, IL).

Sample aliquots (100 μg protein) were electrophoresed using 4–12% Bis-Tris NuPAGE mini-gels (Invitrogen, Carlsbad, CA) before electroblotting to nitrocellulose membrane. Next, the membrane was incubated for 1 h at 25 °C in blocking buffer consisting of 5% skim milk powder in TBST (20 mM Tris–HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.4). The blocked membrane was incubated overnight at 4 °C with either goat HO-1 or rabbit Nrf2 polyclonal antibodies (1:1000 dilution).

After washing repeatedly in TBST on the next day, the membrane was then incubated for 2 h at 25 °C with either anti-goat IgG-HRP or anti-rabbit IgG-HRP (1:100,000 dilution). Finally, after washing repeatedly with TBST, the membrane was incubated with SuperSignal WestFemto Maximum Sensitivity Kit reagents (Pierce, Rockford, IL) for subsequent imaging analysis (Kodak 440 CF image station). Afterwards, the membrane was processed with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and incubated with mouse β-actin monoclonal antibody (1:40,000 dilution) for 2 h at 25 °C. After being washed repeatedly with TBST, the membrane was incubated with anti-mouse IgG-HRP (1:200,000 dilution) for 1 h. Then, the membrane was washed repeatedly with TBST and processed for imaging analysis as before.

2.5. Assay of heme oxygenase activity

The heme oxygenase assay was basically performed as described (Polte et al., 2000). Cells were washed with PBS and pelleted by centrifugation. The cell pellets were then frozen at −80 °C. Upon thawing and sonication of the pellets in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$ and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN), the cell lysates were centrifuged at 18,000*g* for 20 min. The supernatant (700 μg protein) was added to a reaction mixture containing 1 mM NADPH, 2 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, 25 μM hemin, and rat liver cytosol (2 mg protein) in a total volume of 1 ml. After incubation at 37 °C for 1 h in the dark, 1 ml chloroform was added to the samples, which were then vortexed. The bottom chloroform phase containing the product, bilirubin, was analyzed spectrophotometrically (difference in absorbance between 464 and 530 nm). Using an extinction coefficient of 40 mM^{-1} cm^{-1}, heme oxygenase activity was calculated and expressed as pmol bilirubin/h/mg protein.

2.6. Glutathione determination

For the spectrophotometric assay of glutathione (reduced form, GSH), or the total concentration of non-protein thiols of which glutathione is known to make up the vast majority, the cells were harvested from 100 mm petri dishes and pelleted by low-speed centrifugation (50–100 ×*g*, 5 min, 20 °C). After supernate removal, the pelleted cells were washed with phosphate-buffered saline and *re*-pelleted. After supernate removal again, the cell pellets were then stored frozen at −80 °C. The next day, each cell pellet was sonicated after adding 0.35 ml of 50 mM Tris/1 mM EDTA buffer, pH 6.8. Then, the whole cell lysates were centrifuged (21,000 ×*g*, 10 min, 4 °C). The supernates were collected and portions adjusted to contain 0.375 mg protein in a volume of 0.3 ml. To each portion was added 0.01 ml of 30% trichloroacetic acid, followed by mixing and incubation in crushed ice for