**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.

Key words: deferoxamine; heme oxygenase-1; iron; phenethyl isothiocyanate

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 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 5A 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 pre-treating them with 10-100 μ M of either desferrioxamine (DFO) or 2', 2'-dipyridyl (DPD) for 16-24 h, before incubating these iron-deficient cells with 0-10 μ M 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 μ M ferrous sulfate for either 4 or 8 h, before incubation with PEITC. In other experiments, HCT116 cells were pre-treated with 10 mM of either N-acetylcysteine or glutathione for 2 h, and also were pre-treated with 2-20 μ M diphenyleneiodonium (DPI), 1-20 mM Tiron, 0.1-1 mM apocynin, or 1-2 μ M rotenone for 0.5-1 h, before incubating these cells with 10 μ M 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, TTGAGGCTGAGCCAGGAACAGAGT. 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 μ g 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 (1:1,000 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 MgCl₂ and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN), the cell lysates were centrifuged at 18,000 *g* for 20 min. The supernatant (700 μ g protein) was added to a reaction mixture containing 1 mM NADPH, 2 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, 25 μ M 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 $40 \text{ mM}^{-1} \text{ cm}^{-1}$, 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 ($50 \times g$, 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 ($21,000 \times g$, 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 30 min. After centrifugation as before, 0.1 ml aliquots of the supernates and also glutathione standards (ranging from 0 - 20 nmol) were separately mixed with 0.9 ml of working Ellman's reagent (0.3 ml of 10 mM 5,5'-Dithiobis(2-nitrobenzoic acid) dissolved in absolute ethanol and combined with 13.2 ml of 0.25 M Tris/20 mM EDTA buffer, pH 8.2). After incubation at room temperature for 20 min, the absorbance of all the samples was measured at 412 nm.

2.7. Microscopic examination of nuclear Nrf2 expression

After cells were cultured and experimentally treated in LabTek chamber slides, they were fixed with 4% formaldehyde in PBS at 25°C for 15 min. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS at 4°C for 5 min. After blocking the slide wells with 1% bovine

serum albumin in PBS at 25°C for 3 h, the cells were incubated overnight at 4°C with rabbit Nrf2 polyclonal antibody (1:500 dilution). After washing repeatedly with PBS on the following day, the cells were next incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1,000 dilution) for 2 h, followed by counterstaining with 4',6-diamidino-2-phenylindole (DAPI). After extensive washing with PBS, the cells were viewed on an Olympus BX60 fluorescence microscope using FITC and UV filters.

3. Results

3.1 *Upregulation of HO-1 expression by PEITC and impact of iron depletion*

As a bioactive class of phytochemicals, isothiocyanates are known to influence gene expression [Abdull Razis et al., 2010; Saw et al., 2011; Prawan et al., 2008]. To determine if a particular isothiocyanate can influence the expression of a certain cytoprotective gene in a specific cell line, HO-1 expression was ascertained in HCT116 cells that were incubated with PEITC in the presence of all of the required nutrients. In essentially a dose-related manner, PEITC increased HO-1 mRNA expression (Figure 1A, left side of image/graph). Next, we asked the critical question whether such PEITC-induced HO-1 upregulation can occur in cells that are deficient in a particular nutrient. To determine if the micronutrient, iron, is important, HCT116 cells were pre-treated with 100 µM DFO for 16 h to chelate and thereby diminish intracellular iron availability before subsequent treatment with PEITC. PEITC at the concentrations tested did not markedly increase HO-1 mRNA expression in the iron-deficient cells (Figure 1A, right side of image/graph). Therefore, these findings suggest that adequate iron is needed by HCT116 cells to enable PEITC to upregulate HO-1 mRNA expression. Because 10 µM PEITC produced

the desired experimental effect on HO-1 mRNA expression, this particular concentration was used in all subsequent experiments. To put this chosen concentration into better perspective, human blood plasma concentrations of PEITC and other isothiocyanates can range up to 1-2 μM [Ye et al., 2002; Ji et al., 2003]. However, incubating cells with 1-5 μM of specific isothiocyanates produced intracellular isothiocyanate/dithiocarbamate concentrations of 800-900 μM after only 0.5 h of incubation [Zhang et al., 1998].

To expand the above work with DFO in making HCT116 cells iron-deficient, additional experiments were done to further examine the effects of DFO. Lower concentrations of DFO (25 and 50 μM rather than 100 μM) were used along with a longer incubation time (24 h rather than 16 h) to try to simulate a less severe state of iron deficiency in the cells. The ability of PEITC to increase HO-1 mRNA expression was not weakened by 50 or 25 μM DFO (Figure 1B). On the other hand, somewhat unexpected results were attained in performing Western-immunoblotting analysis. That is, 50 μM , and even 25 μM , DFO took away the ability of PEITC to induce HO-1 protein expression (Figure 1C). The simplest explanation concerning the discrepancy between HO-1 mRNA and HO-1 protein is that the DFO-induced iron depletion caused impairment of HO-1 mRNA translation, or perhaps increased HO-1 protein degradation. An ubiquitin-proteosomal system that is responsible for HO-1 protein turnover has been reported [Lin et al., 2008]. In any event, HO-1 protein is apparently more sensitive than HO-1 mRNA in being affected by iron deficiency in PEITC-treated HCT116 cells. This particular finding is significant because it is the gene product (protein) that ultimately acts to function in cells. In the case of HO-1 protein, this gene product functions as an enzyme to produce products having anti-oxidative and anti-inflammatory properties [Paine et al., 2010].

To see if another iron chelator would have a similar effect as DFO, HCT116 cells were pre-treated with 2,2'-dipyridyl (DPD) and then treated with PEITC. DPD also weakened the ability of PEITC to increase the expression of HO-1 mRNA (Figure 2A) and HO-1 protein (Figure 2B), although DPD was not as effective as DFO in this regard. However, DFO and DPD do differ outside of being high affinity chelators of iron. DFO is known to be hydrophilic and binds ferric ions, whereas DPD is known to be hydrophobic and binds ferrous ions. Because of the greater effectiveness of DFO over DPD in weakening the upregulatory effect of PEITC on HO-1 expression, DFO received greater emphasis in subsequent experiments.

3.2 Iron-mediated reversibility of the weakened PEITC-induced HO-1 upregulation caused by the iron chelators

If the effect of DFO was actually a result of the chelation of iron and not any other divalent metal ion, replenishment of iron would be expected to reverse the iron deficiency-inducing effect of DFO in weakening PEITC-induced HO-1 upregulation. To determine if the weakening effect of iron depletion on PEITC-induced HO-1 upregulation can be reversed, HCT-116 cells made iron-deficient with DFO were treated with ferrous sulfate (FeSO_4) to replenish the cells with iron. The iron-replenished cells were then incubated with PEITC, for subsequent determination of HO-1 expression. Iron replenishment for 4 h largely restored the ability of the iron-depleted cells to fully express HO-1 mRNA upon incubation with PEITC (Figure 3A). There was complete restoration of PEITC-induced HO-1 mRNA upregulation in iron-depleted cells with a longer iron replenishment time of 8 h (Figure 3B). Interestingly, the cells that were made iron-deficient had a rather pale flattened appearance, but this abnormal morphology was corrected with FeSO_4 treatment (data not shown).

In hindsight, the above experiments were broadened by including additional treatments of the cells, in order to also determine any possible effects of FeSO₄ alone, DFO plus FeSO₄, and FeSO₄ plus PEITC, on the expression of HO-1 mRNA (Figure 3C). FeSO₄ alone (gel lane 4) and DFO plus FeSO₄ (gel lane 5) did not influence HO-1 mRNA expression. Furthermore, FeSO₄ did not affect, or potentiate, the upregulation of HO-1 mRNA expression caused by PEITC (gel lane 7). Consistent with all of the HO-1 mRNA data are the complementary findings showing the expression of HO-1 protein (Figure 3D). Most notable, PEITC-induced upregulation of HO-1 protein expression in HCT116 cells was weakened by prior DFO-induced iron depletion, but again, this weakening effect of DFO on PEITC-induced HO-1 protein induction was completely rectified with iron replenishment.

The HO-1 gene product functions as an enzyme to degrade heme, thereby producing biliverdin, ferrous iron, and carbon monoxide. These products have anti-oxidative and anti-inflammatory properties [Paine et al., 2010]. Therefore, to extend the HO-1 protein expression findings, the enzymatic activity of heme oxygenase was assayed in experiments designed to look at the potential induction of heme oxygenase activity by PEITC and the impact of relatively milder iron depletion and replenishment. PEITC increased heme oxygenase activity in HCT116 cells having adequate iron, but this induced effect of PEITC was weakened in cells that were pre-treated with DFO to make them iron-deficient prior to PEITC treatment (Figure 4A). However, this weakened induction of heme oxygenase activity was largely eliminated in the iron-deficient cells after iron replenishment (Figure 4B).

To see if the ability of another isothiocyanate to upregulate HO-1 is affected by iron deficiency, fundamental experimentation was performed with sulforaphane (SFN). Similar results were obtained when SFN was tested in place of PEITC in similar experiments. More

specifically, SFN-induced upregulation of HO-1 mRNA expression in HCT116 cells was weakened by prior DFO-induced iron depletion, but this weakening effect of DFO on SFN-induced HO-1 mRNA upregulation was largely rectified with 4 h of iron replenishment (Figure 5A), or completely rectified with 8 h of iron replenishment (Figure 5B).

3.3 *Impact of glutathione on HO-1 expression and effect of iron depletion on PEITC-induced lowering of glutathione*

Intracellular concentrations of glutathione, particularly the reduced form (GSH), can influence the expression of genes that are redox-sensitive [Tanaka et al., 1997], which is characteristic of HO-1. Lowering of GSH levels would be expected to eventually result in redox-sensitive gene activation. Therefore, the effect of PEITC on reduced glutathione levels was determined in HCT116 cells to ascertain a possible link between GSH and induction of HO-1 expression by PEITC. PEITC decreased glutathione in a dose-related manner (Figure 6A). To determine if this lowering of glutathione is important for the upregulation of HO-1 caused by PEITC, experiments were done to examine the effects of exogenously-added glutathione (GSH) and N-acetylcysteine (NAC) on PEITC-induced HO-1 upregulation. When cells were pretreated with either GSH or NAC for 2 h, but even 1 h, and then treated with PEITC, the upregulation of HO-1 mRNA expression (Figure 6B) and also HO-1 protein expression (Figure 6C) caused by PEITC were both prevented. These findings would imply that the GSH and NAC acted to preserve intracellular glutathione, thereby stopping thiol redox-sensitive activation of the HO-1 gene.

It is conceivable that DFO weakened the capability of PEITC to upregulate HO-1 expression by preventing PEITC from lowering glutathione levels. Thus, to determine whether

iron is needed to enable PEITC to lower glutathione levels, HCT116 cells were made iron-deficient with DFO and then incubated with PEITC before measuring the levels of glutathione. In looking at the outcome (Figure 7A), it was unexpectedly found that DFO by itself lowered glutathione levels to some extent, which was further confirmed in an expanded experiment with DFO (Figure 7B) and using the other iron chelator, DPD (Figure 7C). When HCT116 cells were incubated with DFO for 2-h intervals up to 8 h, glutathione levels were unaffected (data not shown), which would indicate that no covalent bonding occurs between glutathione and DFO. Nonetheless, as lastly seen in Figure 7A, glutathione levels in the DFO-induced, iron-deficient cells were further lowered by PEITC, which would imply that iron is unimportant for the glutathione-lowering effect of PEITC or that PEITC does not depend on iron in lowering glutathione levels.

3.4 Effect of iron depletion and replenishment on PEITC-induced augmentation of Nrf2

One of the primary transcription factors known to activate the HO-1 gene is nuclear factor erythroid 2-related factor 2 (Nrf2) [Alam et al., 1999]. Once activated in the cytoplasm with an increase in half-life, Nrf2 translocates into the nucleus in order to transcriptionally activate responsive genes, including HO-1 [Prawan et al., 2008]. Increased Nrf-2 abundance and nuclear localization would be expected to increase HO-1 expression, whereas decreased Nrf-2 abundance and nuclear localization would be expected to decrease HO-1 expression. To examine whether Nrf2 was affected in iron-sufficient versus iron-deficient HCT116 cells that were both incubated with PEITC, Western-immunoblotting analysis of Nrf2 in whole cell lysates was performed. As can be seen (Figure 8A), PEITC increased Nrf2 abundance, but DFO-induced iron depletion reduced the ability of PEITC to increase Nrf2 abundance. However, iron

replenishment (via FeSO₄) restored the ability of PEITC to increase Nrf2 abundance. To determine the cellular localization of Nrf2 under these experimental conditions, immunocytofluorescence microscopy was performed. Based on the microscopic images (Figure 8B), PEITC increased the presence of Nrf2 in nuclei, as indicated by the more intense green fluorescence. In contrast, DFO-induced iron depletion weakened the ability of PEITC to increase nuclear Nrf2 presence, but this weakening was overcome or reversed with iron replenishment.

3.5 Comparative effect of PEITC with ethacrynic acid

As an electrophilic compound, PEITC can react with the reduced form of glutathione (GSH) to produce a glutathionyl-PEITC conjugate [Zhang et al., 1995]. Other electrophilic compounds can also react with GSH. For example, ethacrynic acid has been found to react with glutathione to produce a glutathionyl-ethacrynic acid conjugate [Awasthi et al., 1993]. Based on its similar reactivity as PEITC with GSH, it seems plausible that ethacrynic acid might affect HO-1 expression similarly as PEITC. To examine this possibility with the fundamental intention of further implicating glutathione in the modulation of HO-1 gene expression, core experiments as done above with PEITC were performed with ethacrynic acid. Using a higher test concentration than was used for PEITC, ethacrynic acid was able to lower glutathione levels in HCT116 cells (Figure 9A). Therefore, it was of interest to comparatively ascertain to some extent whether ethacrynic acid affects HO-1 and Nrf2 in a manner like PEITC and if so to examine the impact of iron depletion. Ethacrynic acid had a similar effect as PEITC in that ethacrynic acid increased HO-1 mRNA expression and this effect was weakened by the iron depletion caused by DFO (Figure 9B). Also, ethacrynic acid had a similar effect as PEITC in increasing the presence of Nrf2 in nuclei of the cells (Figure 9C). Moreover, it can be seen that

this ethacrynic acid-induced accumulation of nuclear Nrf2 was weaker in cells that had been made iron-deficient with DFO, but this situation was reversed with FeSO₄ that replenished the iron-deficient cells with iron. Overall, these effects of ethacrynic acid in iron-sufficient and iron-deficient cells are consistent with the effects of PEITC, with the common observation that both compounds lower intracellular glutathione.

3.6 Possible involvement of superoxide in PEITC-induced upregulation of HO-1

Superoxide can act as a signaling oxidant [Buetler et al., 2004] to help mediate redox-sensitive gene activation. Heme iron-dependent NADPH oxidase catalyzes a reaction that produces superoxide [Groemping and Rittinger, 2005], which can be converted to hydrogen peroxide. Reactive oxygen species (ROS) have been shown to cause upregulation of HO-1 expression [Guyton et al., 1996]. One proposed model [Dinkova-Kostova et al., 2002] is that ROS enhance the availability of activated Nrf2, which would favor HO-1 gene activation. To examine if superoxide might be involved in PEITC-induced HO-1 upregulation, the effects of a superoxide scavenger (Tiron) and two known inhibitors (diphenyleneiodonium and apocynin) of the superoxide-producing enzyme, NADPH oxidase, on PEITC-induced HO-1 upregulation were determined. All three of these test compounds inhibited PEITC-induced upregulation of HO-1 protein expression (Figure 10A). Also, the inhibitory effects of diphenyleneiodonium (Figure 10B) and apocynin (Figure 10C) were dose-related, but the inhibitory effect of Tiron was not (Figure 10D). Another experiment indicated that superoxide from mitochondria was not involved in the upregulation of HO-1 mRNA expression caused by PEITC, since rotenone, which at the concentration tested is known to inhibit mitochondrial electron transfer, was without an effect (Figure 10E).

Because it showed the most potent effect, DPI was further studied in immunocytofluorescence microscopy-based experiments to see if DPI had an effect on the presence of nuclear Nrf2 as affected by PEITC. As before, PEITC increased the presence of Nrf2 in nuclei of HCT116 cells, but this effect of PEITC on Nrf2 was inhibited by DPI (Figure 10F). This observation would suggest that DPI inhibited NADPH oxidase so that the production of superoxide or downstream metabolite was insufficient to allow for ROS-mediated Nrf2 activation and subsequent nuclear translocation.

4. Discussion

The observation that PEITC increases HO-1 expression in HCT116 cells is consistent with a previous study [Prawan et al., 2008], which found that many different kinds of isothiocyanates upregulate HO-1 gene transcription. In addition to this existing knowledge, the central findings in the current study support the novel view that adequate amounts of the metal micronutrient, iron, is critical for enabling the isothiocyanate, PEITC, to fully induce HO-1 expression. The importance of iron was ascertained by utilizing two distinct iron chelators to essentially create a state of cellular iron deficiency. DFO is hydrophilic and chelates intracellular iron such as that localized in the endosomal and lysosomal compartments [Doulias et al., 2003]. DPD is hydrophobic and partitions into the plasma membrane, where it confiscates iron from the transferrin/transferrin receptor complex [Nunez et al., 1983] that normally functions in the acquisition of extracellular iron. Thus, when iron was made unavailable in HCT-116 cells by using either DFO or DPD, the strong potential of PEITC to upregulate HO-1 was weakened. This weakening effect by DFO and DPD was reversed when the iron-depleted cells were treated with FeSO_4 to overcome the iron-deficient state.

In explaining how iron seemingly plays a role in enabling HCT116 cells to fully express HO-1 when the cells are stimulated with PEITC, the possible involvement of iron in facilitating the production of ROS needs to be considered. It is well known that iron is involved in forming ROS to mediate cell signaling pathways [Galaris and Pantopoulos, 2008]. PEITC can induce oxidative stress, as evidenced by greater ROS production in cells that have been incubated with PEITC [Xiao et al., 2006; Powolny and Singh, 2010]. We recently reported that DFO inhibited the induction of ROS production in PEITC-treated HCT116 cells [Hoffman et al., 2015], which would suggest an involvement of iron in facilitating the production of ROS as caused by PEITC. Particular ROS, such as superoxide [Buetler et al., 2004] and hydrogen peroxide [Stone and Yang, 2006], are capable of acting as signaling molecules for mediating the activation of redox-sensitive genes, of which HO-1 is a prime example [Ryter and Choi, 2002]. It is conceivable that chelation of iron in HCT116 cells by DFO would subsequently reduce the availability of iron needed to help generate proper levels of ROS by NADPH oxidase for subsequent HO-1 gene upregulation as caused by PEITC.

Also in need of consideration is the crucial role played by iron in the function of membrane-bound NADPH oxidase in producing superoxide. An isoform of NADPH oxidase is present in colon epithelial cells and produces ROS to mediate signal transduction [Park et al., 2004], which can lead to redox-sensitive gene activation. Other data reported in our study would imply that superoxide is important in mediating the HO-1 upregulation caused by PEITC. Tiron, which is a superoxide scavenger, was found to inhibit PEITC-induced HO-1 upregulation. Moreover, diphenyleneiodonium and apocynin, which are known inhibitors of NADPH oxidase that catalyzes the formation of superoxide, were also found to inhibit PEITC-induced HO-1 upregulation. Iron is apparently important for the functioning of NADPH oxidase, as

concluded in a human study. Neutrophil NADPH oxidase activity was lower in iron-deficient human subjects than iron-sufficient subjects, but treatment with iron supplementation normalized the low NADPH oxidase activity [Kurtoglu et al., 2003]. This clinical observation supports the notion that iron is required for NADPH oxidase to have normal enzymatic activity that can produce a signaling oxidant. In the present study, it could be that DFO chelated much of the intracellular iron in HCT116 cells, and thus made proper amounts of iron unavailable for incorporation in the heme moiety of flavocytochrome B558, which is a critical working component of a membrane-bound subunit of NADPH oxidase [Groemping and Rittinger, 2005]. As a pivotal element in flavocytochrome B558, iron facilitates the transfer of electrons from NADPH to molecular oxygen to produce superoxide [Zhu and Silverman, 2008].

The oxidative stress known to be induced by PEITC [Xiao et al., 2006; Powolny and Singh, 2010; Hoffman et al., 2015] and the resulting upregulation of HO-1 expression could be the consequence of an effect of PEITC on intracellular glutathione, which is an important thiol antioxidant. When HCT116 cells were incubated with PEITC, the levels of glutathione were lowered. Via electrophilic addition, PEITC can react with the sulfhydryl group of glutathione, resulting in the formation of a glutathionyl-PEITC conjugate [Zhang et al., 1995]. Hence, the depletion of glutathione in this manner would probably allow normally-produced ROS to accumulate, which in turn would create the stressful conditions for increasing HO-1 expression. On the other hand, merely lowering intracellular glutathione does not appear to be adequate for triggering upregulation of HO-1 expression. PEITC, ethacrynic acid, and DFO were each found to lower the levels of glutathione in HCT116 cells, although it is unclear how DFO decreased glutathione. DFO is not known to be strongly electrophilic unlike PEITC and ethacrynic acid, meaning that DFO would not be expected to react covalently with glutathione. Yet, despite their

similar glutathione-lowering effects, only PEITC and ethacrynic acid caused an accompanying increase in HO-1 expression. It could be that PEITC and ethacrynic acid, but not DFO, eventually targets a specific sulfhydryl-containing protein after glutathione has been diminished to a certain point. This sulfhydryl-containing protein is critically involved in the regulation of HO-1 gene activation as described below.

Nrf2 plays a major role in HO-1 gene activation. Within the promoter region of the HO-1 gene is a stress response element for binding of the basic leucine zipper transcription factor, Nrf2 [Alam et al., 1999; Alam and Cook, 2003]. However, Nrf2 must first be dissociated from the sulfhydryl-containing protein, Keap1, which is thought to be a molecular sensor for electrophilic compounds that induce expression of cytoprotective genes [Dinkova-Kostova et al., 2002]. Essentially, ROS can oxidize the sulfhydryl groups of key cysteinyl residues in Keap1. On the other hand, electrophilic compounds, which would include PEITC, can react covalently with these sulfhydryl groups in Keap1. In any event, Keap1 undergoes structural modification that permits Nrf2 to become dissociated from Keap1. Nrf2 then translocates from cytoplasm into the nucleus in order to transcriptionally activate responsive genes, including HO-1 [Prawan et al., 2008]. Certain mitogen-activated protein kinases are also involved in Nrf2 activation when cells are incubated with isothiocyanates [Xu et al., 2006]. In the current study, there was stimulation of the translocation or accumulation of Nrf2 in the nuclei of HCT116 cells that were incubated with PEITC. This stimulatory effect did not occur in iron-deficient cells when incubated with PEITC, but this situation was rectified with iron replenishment. Therefore, the lack of availability of intracellular iron that was created by DFO apparently resulted in a situation that was not conducive for Nrf2 activation and subsequent nuclear translocation as caused by PEITC.

It is conceivable that once PEITC has depleted glutathione to a threshold level in HCT116 cells, Keap1 then becomes the target of PEITC. There is evidence that isothiocyanates can react with the cysteinyl sulfhydryl groups in Keap1 [Dinkova-Kostova et al., 2002]. By providing PEITC with excess amounts of an alternative sulfhydryl-containing target, N-acetylcysteine was found to inhibit PEITC-induced HO-1 upregulation. Thus, N-acetylcysteine probably abrogated the effect of PEITC on HO-1 expression by electrophilic addition so that perhaps insufficient amounts of PEITC were available to react with Keap1. However, the ability of N-acetylcysteine to act as an antioxidant against PEITC-induced ROS and subsequent oxidation of the cysteinyl thiol groups in Keap1 cannot be ignored.

In conclusion, the hormetic phytochemical, PEITC, depends on the metal micronutrient, iron, in order to have full strength in stimulating the cytoprotective gene, HO-1. In other words, adequate availability of the micronutrient, iron, in cells is required to enable the phytochemical, PEITC, to fully upregulate HO-1.

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FIGURE LEGENDS

Figure 1. Reduced ability of PEITC to upregulate HO-1 expression in HCT-116 cells made iron-deficient with the iron chelator, deferoxamine (DFO). The cells were pre-treated with 100 μ M deferoxamine (DFO) for 16 h (**A**), or with 25 and 50 μ M DFO for 24 h (**B** and **C**), before treating the cells with 10 μ M PEITC for 5 h. Using gene specific primers for the target gene, HO-1, and the internal control gene, β -actin, multiplex relative RT-PCR analysis was performed (**A** and **B**). Relative HO-1 mRNA values are the average $\pm$ SEM. Additionally, Western immunoblotting analysis was done (**C**). All of the results are representative of 3-4 separate experiments.

Figure 2. Reduced ability of PEITC to upregulate HO-1 expression in HCT-116 cells made iron-deficient with the iron chelator, 2', 2'-dipyridyl (DPD). The cells were pre-treated with 25, 50, 100 and 200 μ M DPD for 16 h (**A**) or with 25, 50, 100 μ M DPD for 24 h (**B**), before treating the cells with 10 μ M PEITC for 5 h. Using gene specific primers for the target gene, HO-1, and the internal control gene, β -actin, multiplex relative RT-PCR analysis was performed (**A**). Relative HO-1 mRNA values are the average $\pm$ SEM. Additionally, Western immunoblotting analysis was done (**B**). All of the results are representative of 3-4 separate experiments.

Figure 3. Rectification by iron replenishment of the reduced ability of isothiocyanates to upregulate HO-1 expression in DFO-treated HCT-116 cells. The cells were pre-treated with DFO for 16 h and then treated during the recovery phase with FeSO_4 for either 4 h (**A** and **C**) or 8 h (**B**), before being incubated with PEITC for 5 h. Multiplex relative RT-PCR analysis was

then performed using gene specific primers for the target gene, HO-1, and the internal control gene, β -actin. Relative HO-1 mRNA values are the average $\pm$ SEM. Additionally, whole cell lysates were prepared for Western immunoblotting analysis (**D**). All of the results are representative of 3-4 separate experiments.

Figure 4. Weaker induction of HO-1 enzyme activity by PEITC in cells made iron-deficient with DFO that can be largely overcome with iron replenishment. In **A**, HCT-116 cells were pre-treated with either 10 or 25 μ M DFO for 24 h and then treated with 10 μ M PEITC for 5 h. In **B**, cells were pre-treated with 25 μ M DFO for 24 h and then replenished with 50 μ M FeSO_4 for 4 h before treating the cells with 10 μ M PEITC for 5 h. Heme oxygenase activity was assayed spectrophotometrically. Values are the average $\pm$ SEM for three separate experiments.

Figure 5. Rectification by iron replenishment of the reduced ability of the related isothiocyanate, sulforaphane (SFN), to upregulate HO-1 expression in DFO-treated HCT-116 cells. The cells were pre-treated with DFO for 16 h and then treated with FeSO_4 during the recovery phase for either 4 h (**A**) or 8 h (**B**), before being incubated with SFN for 5 h. Multiplex relative RT-PCR analysis was then performed using gene specific primers for the target gene, HO-1, and the internal control gene, β -actin. Relative HO-1 mRNA values are the average $\pm$ SEM. All of the results are representative of 3-4 separate experiments.

Figure 6. Lowering of intracellular glutathione by phenethylisothiocyanate (PEITC) and effect of glutathione modulators on HO-1 expression in HCT-116 cells. In **A**, cells were incubated with a certain concentration range (0-10 μ M) of PEITC. After 2 h of incubation, glutathione was

determined spectrophotometrically. Also, cells were pre-treated with 10 mM of either glutathione (GSH) or N-acetylcysteine (NAC) for 1-2 h and then treated with 10 μ M PEITC for 5 h, followed by multiplex relative RT-PCR analysis (**B**). Relative HO-1 mRNA values are the average $\pm$ SEM. Also, Western immunoblotting analysis was done (**C**). All of the results are representative of 3-4 separate experiments.

Figure 7. Effect of iron chelators on glutathione levels in PEITC-treated and untreated HCT116 cells. The cells were pre-treated with 100 μ M DFO for 16 h and then treated, or not treated, with 10 μ M PEITC for 5 h (**A**). Additionally, cells were incubated with various concentrations of just either DFO (**B**) or DPD (**C**) for 20 h. Afterwards, glutathione was determined spectrophotometrically. Values are the average $\pm$ SEM for 4 separate experiments.

Figure 8. Impact of iron deletion on PEITC-induced augmentation of Nrf2 in HCT116 cells. The cells were incubated with 10 μ M PEITC for 5 h. Also, cells were pre-treated with 100 μ M DFO for 16 h, and then either treated or not treated with 200 μ M FeSO₄ for 4 h, before being incubated with PEITC. Whole cell lysates were prepared for Western immunoblotting analysis (**A**). Additionally, immunocytofluorescence microscopy was performed to visualize cellular localization of Nrf2 (**B**). Nuclei were visualized by staining DNA with DAPI, whereas Nrf2 was co-visualized with an Nrf2 antibody/AlexaFluor 488 detection system. All of the results are representative of 3-4 separate experiments.

Figure 9. Effect of ethacrynic acid on glutathione levels, HO-1 expression, and Nrf2 localization in HCT116 cells and impact of iron depletion. In **A**, cells were treated with 50 μ M

ethacrynic acid (EA), or 10 μ M PEITC for comparison. After 2 h of incubation, glutathione levels were determined spectrophotometrically. In **B**, cells were pre-treated with DFO (ranging from 0-100 μ M) for 24 h and then treated with 50 μ M EA for 5 h. Multiplex relative RT-PCR analysis of HO-1 mRNA was then performed. In **C**, cells were incubated with 50 μ M EA for 5 h. Also, cells were pre-treated with 100 μ M DFO for 16 h, and then either treated or not treated with 200 μ M FeSO₄ for 4 h, before being incubated with EA. Immunocytofluorescence microscopy was then done to visualize cellular localization of Nrf2. Nuclei were visualized by staining DNA with DAPI, whereas Nrf2 was co-visualized with an Nrf2 antibody/AlexaFluor 488 detection system. All of the results are representative of 3-4 separate experiments. Measurements of glutathione and relative HO-1 mRNA are the average $\pm$ SEM.

Figure 10. Effect of diphenyleneiodonium (DPI), apocynin, Tiron, and rotenone on HO-1 upregulation and nuclear translocation of Nrf2 as both caused by PEITC. HCT-116 cells were pre-treated with DPI, apocynin, Tiron, or rotenone for 1 h. The cells were then treated with PEITC for 5 h, followed by determination of HO-1 protein (**A, B, C, D**) or HO-1 mRNA (**E**). Also, immunocytofluorescence microscopy was performed to visualize cellular localization of Nrf2 (**F**). Nuclei were visualized by staining DNA with DAPI, whereas Nrf2 was co-visualized with an Nrf2 antibody/AlexaFluor 488 detection system. All of the results are representative of 3-4 separate experiments.

Figure 1

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