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The Effects of Equal Doses of Sulforaphane, Curcumin and Quercetin on Heme Oxygenase-1 Gene and Protein Expression in Mice Liver

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Abstract: Heme Oxygenase-1 (HO-1) is an enzyme that possess anti-oxidant, anti-inflammatory and cytoprotective functions. Induction of HO-1 occurs as an adaptive and beneficial response to various injurious stimuli such as oxidative stress. This study aimed at monitoring the effects of administration of equal doses (50 mg/kg) of sulforaphane, curcumin and quercetin for 14 days on the levels of liver HO-1 gene and protein expression in mice. The 24 ICR male white mice (25-30 g) were divided into 4 groups: the control (vehicle treated) group (n = 6) the sulforaphane treated group (n = 6), the quercetin treated group (n = 6) and the curcumin treated group (n = 6). The chemicals were dissolved in a vehicle (DMSO, Tween-20 and normal saline in the ratio of 0.05: 0.1: 0.85) and administered intraperitoneally at a dose of 50 mg/kg for 14 days. Only the vehicle was administered to the control group. At day 15, animals were sacrificed and their livers isolated. Total RNA was extracted, reverse transcribed and subjected to quantitative real-time PCR to detect HO-1 gene expression. Agarose gel electrophoresis was also performed to verify amplification specificity. HO-1 protein expression was determined by Western blotting. HO-1 gene expression showed significant increase of 4.6, 3.3 and 3.0-fold and HO-1 protein expression showed significant increase of 2.3, 1.8 and 1.7-fold following treatment with sulforaphane, curcumin and quercetin respectively. Sulforaphane had the highest impact on the induction of HO-1 expression in mice liver, followed by curcumin and quercetin.

Key words: Heme oxygenase-1, gene expression, protein expression, sulforaphane, quercetin, curcumin

INTRODUCTION

It is established that a diet rich in fruits and vegetables provides protection against cancer (Stan *et al*., 2008). They contain antioxidants which promote the removal of reactive species generated during normal oxidative metabolism and unwanted xenobiotic chemicals (Abuajah *et al*., 2015). Some chemicals found in food and phenolic antioxidants are chemoprotective, i.e., they induce the expression of genes and proteins involved in cellular defence. The increased expression of cellular defence proteins provided added protection against oxidative/chemical stress. Some of these proteins belong to phase 2 drug metabolizing enzymes, although, other enzymes and antioxidant proteins were also involved. These proteins are known as phase 2 proteins (Keum, 2012). Phase 2 proteins are mainly regulated by the Nrf2/ARE system (Kitteringham *et al*., 2010; Abdullah *et al*., 2012). Heme Oxygenase-1 (HO-1) is an example of phase II proteins regulated by Nrf2 (He *et al*., 2001). HO-1 is a rate-limiting enzyme that catalyzes the degradation of heme (a pro-oxidant) to carbon monoxide, biliverdin and free iron (Keum *et al*., 2006). HO-1 induction is important in terms of cellular defense mechanism due to the fact that HO-1 expression is inducible in response to various forms of cellular insult. Moreover, the end products of HO-1 catabolism exhibit anti-oxidative, anti-inflammatory and anti-apoptotic properties (Keum *et al*., 2006). The objective of this study is to determine the nature and potency of HO-1 expression in mice liver induced by equal doses (50 mg/kg) of several chemicals commonly found in diet, i.e., sulforaphane, curcumin and quercetin.

MATERIALS AND METHODS

Chemicals and reagents Primers were purchased from Vivantis Technologies (Oceanside, CA, USA). TRIzol

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reagent was purchased from Life Technologies (Carlsbad, California, USA). iScript™ cDNA Synthesis kit and iQ™ SYBR® Green Supermix (2X) were purchased from Bio-Rad (Hercules, California, USA). Sulforaphane was purchased from Santa Cruz Biotechnology (Paso Robles, California, USA). Gel Red Nucleic Acid Gel Stain (10,000 X in water) was purchased from Biotium (Hayward, California, USA). Sulforaphane was purcased from Santa Cruz Biotechnology (Paso Robles, California, USA). Curcumin, quercetin and all other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). RIPA buffer and anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology (Dallas, USA). Chemilumine scence Western blotting detection reagents were purchased from Amersham (Uppsala, Sweden). Nitrocellulose membrane was purchased from Sigma-Aldrich (Seelze, Germany). HO-1 mouse polyclonal primary antibody and β-actin rabbit polyclonal antibody were purchased from Abcam Biotechnology (Cambridge, UK).

Animal treatment: About 24 adult male ICR white mice (25-30 g) were divided into 4 groups: 1 sulforaphane treated group (n = 6), 2 quercetin treated group (n = 6) 3 curcumin treated group (n = 6) and 4 control (vehicle treated) group (n = 6). A vehicle (DMSO, Tween 20 and normal saline at a ratio of 0.05: 0.1: 0.85) was used to dissolve sulforaphane, quercetin and curcumin. The vehicle (DMSO, Tween 20 and normal saline in the ratio of 0.05: 0.1: 0.85) was similarly administered to the control group. All chemicals were administered intraperitoneally (i.p.) at a dose of 50 mg/kg body weight for 14 days. At day 15, the animals were sacrificed and their livers isolated. The animal study protocol was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) and the appoval code is: FP/FAR/2012/AZMAN/23-MAY/442-JUNE-2012-JUNE-2015.

RNA extraction: Total RNA from frozen liver tissues was isolated using TRIzol reagent, according to the manufacturer's instructions. Isopropyl alcohol (Sigma, USA) was added in each extraction step to precipitate the total RNA. Extracted total RNA pellet was then washed with 75% ethanol and dried before being dissolved in RNase free water. Total RNA was stored at -80°C immediately after extraction. Concentration and purity of the extracted RNA were determined by NanoDrop spectro photo meter 2000 c (Thermo scientific, USA) at a wavelength of 260 nm (OD260). RNA with RNA Integrity Number (RIN) ranging from 7-10 and absorbance ratio of A260-A280 ranging from 1.5-2.0 was used for cDNA synthesis.

Table 1: Primer sequence for GAPDH and HO-1

Gene description	Primer sequence
GADPH	F: 5'-GTGGAGTCTACTGGTGTCTTCA-3'
	R: 5'-TTGCTGACAATCTTGAGTGAGT-3'
HO-1	F: 5′-CCTCACTGGCAGGAAATCATC-3′
	R: 5′-TATGTAAAGCGTCTCCACGAGG-3′

Reverse transcription: Generation of cDNA from RNA was done using iScript cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. Briefly, a volume of total RNA (containing 1 μg) from each sample was added to a mixture of 4 μL of 5X iScript reaction mix, 1 μL of iScript reverse transcriptase and a volume of nuclease-free water in a total volume of 20 μL. The final reaction mix was kept at 25°C for 5 min, 42°C for 30 min and heated to 85°C for 5 min in a thermocycler (TC-412, Techne, Barloworld Scientific, UK). The cDNA was then used as a template for amplification by PCR.

Quantification of HO-1 gene expression by quantitative real-time PCR: Quantitative real-time PCR was performed on the MiniOpticon cycler (Bio-Rad, USA). The total reaction volume used was 20 μL, consisting of 1 μL of 10 μM forward primer and 1 μL of 10 μM reverse primer (500 nM final concentration of each primer), 10.0 μL of iQ™ SYBR® Green Supermix (2X) (Bio-Rad, USA), 6.0 μL of nuclease-free water and 2.0 μL of cDNA. Both forward and reverse primers for the genes of interest in this study were designed according to previous studies and synthesized by Vivantis Technologies (Oceanside, CA, USA). The primer sequences for our gene of interest are shown in Table 1.

The thermo cycling conditions were initiated at 95°C for 30 sec, followed by 40 PCR cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 30 sec. At the end of each cycle, a melting curve (dissociation stage) was performed in order to determine the specificity of the primers and the purity of the final PCR product. All measurements were performed in triplicate and No-Template Controls (NTC) were incorporated onto the same set of PCR tubes to test for the contamination by any assay reagents. Threshold, cycles were determined for each gene and quantification of templates was performed according to the relative standard curve method. The relative gene expression (ΔΔCt) technique as defined in the Applied Biosystems User Bulletin No. 2 (Livak and Schmittgen, 2001) was used to analyse the real-time PCR data. In short, the expression level of each target gene was given as relative amount normalized against GAPDH standard controls. Subsequently, agarose gel electrophoresis was performed to determine the reliability of the melting curve analysis and to confirm the size of the PCR product. Briefly, electrophoresis was performed using 1% agarose gel in

order to separate the real-time PCR products. GelRed™ nucleic acid gel stain (Biotium, USA) was used to stain the gels for 30 min and the gels were subsequently de-stained in distilled water for 30 min. Bands were then visualized under ultraviolet light using a gel documentation system (Fluor Chem FC2, Alpha Innotech, USA).

Preparation of cytosolic protein fraction: Liver tissue samples were homogenized in RIPA lysis buffer (which contained 10 μL PMSF, 10 μL sodium orthovanadate and 10 μL protease inhibitor cocktail solution per 1 mL of 1X RIPA lysis buffer). After centrifugation, the supernatants (cytosolic fractions) were collected and their protein concentrations were determined.

Western blotting: Standard Western blotting procedure was used for immunodetection of proteins. Briefly, 100 μg of liver protein was separated using 15% Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE) electrophoresis. The proteins in the gel were then transferred to nitrocellulose membrane. The membrane was then incubated for 20 min at room temperature in blocking solution [150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.1% (v/v) Tween-20 and 10% non-fat milk powder (pH 7.4)]. After blocking, the membrane were incubated with the following antibodies i.e., primary polyclonal rabbit anti-mouse HO-1 and primary polyclonal rabbit anti-mouse actin for 1 h at room temperature. Subsequently incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody was carried out for another 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence method according to the manufacturer's instructions (Amersham, Uppsala, Sweden). The intensity of the protein bands were quantified, relative to the signals obtained for actin, using ImageJ software.

Statistical analysis: Data are presented as mean± Standard Error of the Mean (SEM). Significant differences between mean values of multiple groups were determined using one-way ANOVA and Student's t-test. Statistical analysis was conducted using the SPSS Software Version 22. The result was considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

The gene expression of HO-1 showed significant increase of 4.6, 3.3 and 3.0-fold following treatment with sulforaphane, curcumin and quercetin as compared to controls ($p<0.05$) (Fig. 1). The protein expression of HO-1 showed significant increase of 2.3, 1.8 and 1.7-fold following treatment with sulforaphane, curcumin and quercetin as compared to controls ($p<0.05$) (Fig. 2).

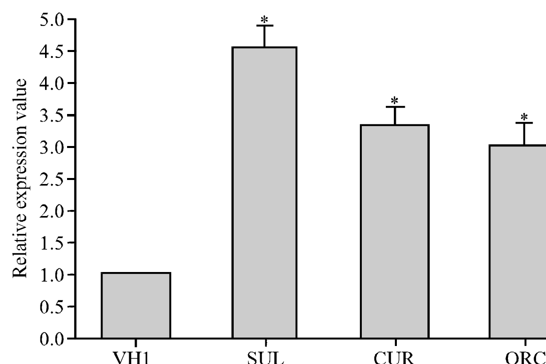

Fig. 1: Effects of intraperitoneal administration of 50 mg/kg sulforaphane, curcumin and quercetin for 14 day on HO-1 gene expression in the livers of mice using real-time PCR (qPCR). Data is presented as mean±SEM. VH1: control (Vehicle treated) group, SUL: Sulforaphane group, CUR: Curcumin group, QRC: Quercetin group. Amplified products were visualized by agarose gel electrophoresis and gene expression was confirmed by identification of the appropriate bands. GAPDH served as a reference gene. *$p<0.05$ compared to controls

HO-1 inhibits oxidative stress by removing excess heme. HO-1 and the catabolic products of heme work in tandem to decrease inflammation and cytotoxic reactions (Chang *et al*., 2015). HO-1 knockout mice are characterized by enlarged spleen and hepatic lesions due to chronic inflammatory process (Choi *et al*., 2011). Humans with HO-1 deficiency have been shown to be susceptible to oxidative stress and inflammation which will eventually lead to severe endothelial damage (Eipel *et al*., 2007). Induction of HO-1 has been shown to prevent hepatocyte damage due to oxidative stress (Brockmann *et al*., 2005). Many of the classical HO-1 triggers have been shown to induce HO-1 expression through nuclear factor E2-related factor 2 (Nrf2) binding to the antioxidant response element (ARE) at the HO-1 promoter region (Martin *et al*., 2004). Experiments conducted on Nrf2-deficient mice showed the importance of Nrf2 in stress-dependent induction of HO-1 because HO-1 was found to be less inducible in such mice (Cho *et al*., 2002). Phytochemicals such as sulforaphane, curcumin and quercetin have been shown to induce HO-1

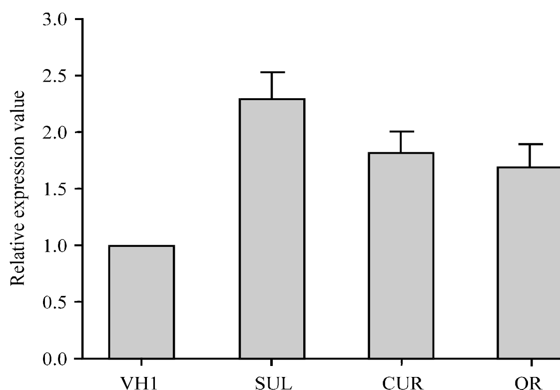

Fig. 2: Effects of intraperitoneal administration of 50 mg/kg sulforaphane, curcumin and quercetin for 14 days on HO-1 protein expression in the livers of mice using Western immunoblotting. Data is presented as mean±SEM. VH1: control (Vehicle treated) group, SUL: Sulforaphane group, CUR: Curcumin group, QRC: Quercetin group. The graph represents the relative amount of HO-1 band density normalized to β-actin. *$p<0.05$ compared to controls

expression in liver cells (hepatocytes) as well as in the liver itself (Yao *et al*., 2007; Bao *et al*., 2010; Noh *et al*., 2015). However, what is not known is which of these phytochemicals is the most potent in inducing HO-1 expression. The results of our study clearly showed that sulforaphane is the most potent in terms of inducing HO-1 expression in mice liver, followed by curcumin and quercetin. Therefore, increased consumption of sulforaphane rich food such as broccoli in humans could be beneficial in terms of general health and cancer chemoprevention strategy. At present, not much can be done to prevent liver cell damage and degeneration. Therefore, induction of HO-1 by safer means such as supplementation with natural products may be an effective strategy to prevent liver carcinogenesis and other liver diseases (Liu *et al*., 2012). A number of chemopreventive agents that exhibit cytoprotective, anti-inflammatory and antioxidant effects by means of induction of HO-1 expression have been identified (Curjuric *et al*., 2010). In addition, our results showed that sulforaphane, quercetin and curcumin were able to induce HO-1 gene expression in mice liver. Therefore, it can be hypothesized that these chemicals induce HO-1 expression in the liver as part of their chemoprevention/chemoprotective effects, however, more studies are needed to confirm this.

CONCLUSION

According to the findings of this study, at a dose of 50 mg/kg for 14 days, administration of sulforaphane has the most significant impact on the induction of the HO-1 expression in the liver of mice, followed by curcumin and quercetin. Sulforaphane can be found abundantly in cruciferous vegetables such as broccoli. HO-1 is transcriptionally upregulated by a large variety of stimuli, e.g., heme, oxidative stress, signaling proteins and organic chemicals. Therefore, the induction of HO-1 by pharmacological means, preferentially through supplementation of various forms of natural products, offers a potential new therapeutic target for liver degenerative diseases. In this respect for some very promising chemoprotective phytochemicals such as sulforaphane, the recommended therapeutic dose which is effective in the prevention or treatment of liver diseases should be aggressively investigated.

RECOMMENDATIONS

The results of this study further strengthens the importance of consuming more fruits and vegetables which could potentially prove to be an affordable chemoprotective measure in the long run. Further studies should be done to conclusively support this strategy.

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