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HEME OXYGENASE 1 GENE EXPRESSION IN MICE LIVER IS DIFFERENTLY AFFECTED BY EQUAL DOSES OF PHYTOCHEMICALS

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A B S T R A C T

Objective: Heme oxygenase (HO-1) is an enzyme that possess anti-oxidant, anti-inflammatory and cytoprotective functions. In case of various harmful stimuli such as oxidative stress, HO-1 is induced as an compatible and helpful response. The purpose of the current study was assessing the impacts of equal doses (50 mg/kg body weight) of sulforaphane, curcumin and quercetin on the expression levels of HO-1 gene. Method:4 groups of ICR male white mice (25–30 g) were formed: group I included the treatment with sulforaphane(n=6), group II included the treatment with quercetin (n=6), group III included the treatment with curcumin (n=6), and group IV was the control (vehicle) group (n=6). The chemicals were applied intraperitoneally at a dose of 50 mg/kg body weight for 14 days. A vehicle (DMSO, tween 20 and normal saline in the ratio of 0.05:0.1:0.85) was similarly applied to the control group. At the 15th day, the animals were killed and their livers were isolated. The total RNA was obtained, the reverse was transcribed and the quantitative real-time PCR was used to assess HO-1 expression. In order to assure the specificity of the amplification, Agarose gel electrophoresis was also conducted. Results: The administration of Sulforaphane caused an increase of 4.6-fold HO-1 proceeded by the treatment with sulforaphane comparing to the control (P<0.05). The treatment with curcumin caused a 3.3-fold induction of HO-1 comparing with the control (P<0.05). The treatment with quercetin led to a 3.0-fold induction of HO-1 comparing with the control (P<0.05). Conclusion: Applying the dose of 50mg/kg body weight, Sulforaphane had the highest influence on inducing of HO-1 in the livers of mice, proceeded by curcumin and quercetin.

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Introduction

It has been established that a diet rich in fruits and vegetables provides protection against cancer [1]. They contain antioxidants which promote the removal of the reactive species generated during the normal oxidative metabolism and unwanted xenobiotic chemicals [2]. Some chemicals found in food (phytochemicals) are chemoprotective i.e. they induce the expression of genes and proteins involved in cellular defence. The increased expression of cellular defence proteins increased the protection against oxidative /chemical stress. Some of these proteins belonged to phase II drug metabolizing enzymes, although other enzymes and antioxidant proteins were also involved. These proteins have been known as phase II proteins [3-5]. The Nrf2/ARE system has been mainly used to regulate Phase II proteins [6, 7]. Heme oxygenase-1 (HO-1) can be mentioned as an example of Phase II proteins which are regulated by Nrf2 [8]. HO-1 is an enzyme that limits the rate, and catalyzes the degradation of heme (a pro-oxidant) to carbon monoxide, biliverdin, and free iron [9]. Considering the cellular defense mechanism, HO-1 induction is significant because HO-1 expression can be induced to respond to different forms of the cellular insult. Additionally, the obtained products of HO-1 catabolism represented anti-oxidative, anti-inflammatory and anti-apoptotic characteristics [9]. The present study aimed at identifying the nature and potency of HO-1 expression induced by the equal doses of several phytochemicals commonly found in diet i.e. sulforaphane, curcumin and quercetin (Table 1).

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Table 1. Structures of phytochemicals involved in this study

Phytochemical	Structure	Structural / Functional classes	Sources
Sulforaphane		Isothiocyanate	Broccoli
Quercetin		Flavonol,1,2,6-triphenol	onions, apples
Curcumin		Diarylheptanioid, Michael acceptor, 1,2-diphenol	Turmeric

Materials and Methods

Chemicals and reagents

Primers were obtained from Vivantis Technologies (Oceanside, CA, USA). TRIzol Reagent was bought from Life Technologies (Carlsbad, California, USA). iScript^{TM} cDNA Synthesis kit and iQ^{TM} SYBR® Green Supermix (2X) were obtained from Bio-Rad (Hercules, California, USA). Sulforaphane was obtained from Santa Cruz Biotechnology (Paso Robles, California, USA). Gel Red Nucleic Acid Gel Stain (10,000X in water) was bought from Biotium (Hayward, California, USA). Sulforaphane was obtained from Santa Cruz Biotechnology (Paso Robles, California, USA). Curcumin, quercetin and all other chemicals were bought from Sigma-Aldrich (St. Louis, Missouri, USA).

Animal treatment

24 adult male ICR white mice (25–30 g) were used as the subjects of the study. They were divided into 4 groups: group I was treated with sulforaphane (n=6), group II was treated with quercetin (n=6), group III was treated with curcumin (n=6), and group IV was the control (vehicle) 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 applied to the control group, in a similar way. All the chemicals were applied intraperitoneally (i.p.) at a dose of 50 mg/kg body weight for 14 days. At the 15th day, the mice were killed, and their livers were isolated. The process of studying the animals was confirmed by the University of Kebangsaan Malaysia Animal Ethics Committee (UKMAEC), and the approval code was: FP/FAR/2012/AZMAN/23-MAY/442-JUNE-2012-JUNE-2015.

RNA Extraction

Total RNA from frozen liver tissues was isolated using TRIzol reagent, based on the instructions provided by the manufacturer. In order to speed up the total RNA, Isopropyl alcohol (Sigma, USA) was added in each extraction level. 75% ethanol was used to wash the extracted total RNA pellet, then the pellet was dried before being dissolved in RNase free water. Total RNA was stored at -80^{0}C immediately after the extraction. NanoDrop spectrophotometer of 2000c (Thermo Scientific, USA) at a wavelength of 260 nm (OD260) was applied to identify the amount of concentration and purity of the extracted RNA. And, to synthesize cDNA, RNA integrity number (RIN) ranging from 7 to 10, and the absorbance ratio of A260 to A280 ranging from 1.5 to 2.0 was administered.

Reverse transcription

IScript cDNA synthesis kit (Bio-Rad, USA) was applied to generate cDNA from RNA based on the instructions provided by the manufacturer. Briefly, in each sample, a volume of total RNA (containing 1 µg) was added to the 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 the 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 1µl of 10µM forward primer and 1µl of 10µM reverse primer (500 nM final concentration of each primer), 10.0 µl of iQ^{TM} SYBR®Green Supermix (2X) (Bio-Rad, USA), 6.0 µl of nuclease-free water and 2.0 µl of cDNA. Both the

forward and reverse primers for the genes examined in the current study were designed based on the examinations done previously, and synthesized by Vivantis Technologies (Oceanside, CA, USA). The primer sequences for the examined gene have been shown in Table 2.

Table 2. 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´

The thermocycling 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. After finishing each cycle, a melting curve (dissociation stage) was done in order to identify the specificity of the primers and the purity of the final PCR product. In order to test the contamination by any assay reagents, all the measurements were conducted in triplicate; and no-template controls (NTC) were administered on the same set of PCR tubes. For each gene, the threshold cycles were identified, and the templates were quantified based on the relative standard curve method. The relative gene expression (ΔΔCt) technique, as defined in the Applied Biosystems User Bulletin No. 2 [10] was used to analyse the real-time PCR data. In short, the expression level of each target gene was given as a relative amount normalized against GAPDH standard controls. Subsequently, agarose gel electrophoresis was conducted to identify the reliability of the melting curve analysis, and 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^{TM} 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 (FluorChem FC2, Alpha Innotech, USA).

Results and Discussion

HO-1 was increased at 4.6-fold, when the mice were treated with sulforaphane comparing with the control (P<0.05). When the mice were treated with curcumin, HO-1 was induced 3.3-fold, comparing with the control (P<0.05). The treatment with quercetin led to 3.0-fold induction of HO-1, comparing with the control (P<0.05) (Figure 1).

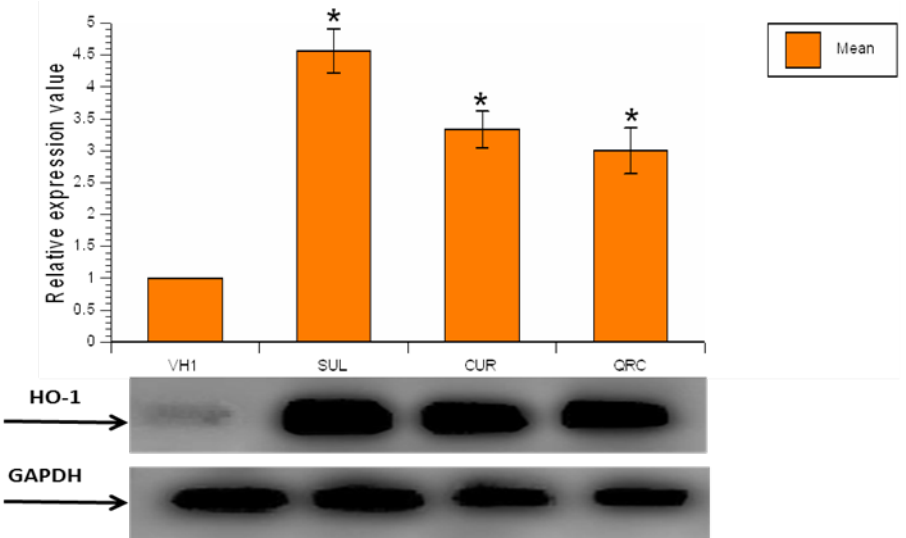

Figure 1: Effects of intraperitoneal administration of 50mg/kg sulforaphane, curcumin and quercetin for 14 days on HO-1 gene expression in the livers of mice. The data was presented as mean ± SEM. VH1: vehicle control group, SUL: sulforaphane group, CUR: curcumin group, QRC: quercetin group.
The amplified products were visualized by agarose gel electrophoresis, and gene expression was confirmed by the identification of the appropriate bands. GAPDH served as a reference gene. * P < 0.05 compared to the control.

HO-1 is transcriptionally upregulated by a large variety of stimuli, including heme, oxidative stress, signaling proteins and organic chemicals. Most of the classical HO-1 stimulants have represented to induce HO-1 expression by nuclear factor of E2-related factor 2 (Nrf2) binding to the antioxidant response element (ARE) at the hemeoxygenase-1 promoter area [11]. The 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 [12]. Phytochemicals such as sulforaphane, curcumin and quercetin have

been shown to induce HO-1 expression in liver cells (hepatocytes) as well as in the liver itself [13-15]. However, what was not known was which of these phytochemicals was the most potent in inducing HO-1 expression. The results of the current study clearly showed that sulforaphane was the most potent phytochemical in terms of inducing HO-1 gene expression in mice liver, followed by curcumin and quercetin. Therefore, the increased consumption of sulforaphane rich food such as broccoli in humans could be beneficial in terms of general health, and cancer chemoprevention strategy.

Conclusion

According to the findings of this study, at a dose of 50mg/kg body weight for 14 days, the administration of sulforaphane represented to have the most significant effect on inducing the HO-1 expression in the livers of mice, proceeded by curcumin and quercetin. Cruciferous vegetables such as broccoli can be considered as the rich source of Sulforaphane. The results of this study further strengthened the importance of consuming more fruits and vegetables which could potentially prove to be an affordable chemoprotective measure. Further studies should be done to conclusively support this strategy.

Conflicts of Interests

The authors did not declare any conflicts of interest.

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