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Correlation between ratio of Nrf2/Keap1 and catalase gene expression in liver of hyperlipidemic rats after administration of 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one

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ABSTRACT

Hyperlipidemia results in excessive superoxide anion radicals that are the cause of oxidative stress. Phytochemical compounds can reduce oxidative stress. The aim of this study was to investigate the correlations between ratio of Nrf2/Keap1 and catalase gene expression in livers of hyperlipidemic rats after administration of 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one. Twenty-four *Rattus norvegicus* rats, aged 8 weeks and weighing an average of 200 g were randomly divided into 6 groups i.e. Group 1 was normal group (N), Group 2 was hyperlipidemic rats (HL), Group 3 was hyperlipidemic rats with simvastatin (HL+SV), and Groups 4-6 were hyperlipidemic rats with 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one doses 10 mg (HL+10), 30 mg (HL+30) or 90 mg/200 g BW (HL+90), respectively, administered orally by gavages. At the end of the study, the rats were euthanized and the livers were used to analyze the ratio of Nrf2/Keap 1 and catalase gene expression. Nrf2/Keap1 ratio and catalase gene expression between groups were analyzed by Kruskal Wallis test. Spearman's correlation test was used to analyze the correlations between Nrf2/Keap1 ratio and catalase gene expression. The administration of 3 different doses of 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one on hyperlipidemic rats increased catalase gene expression. There was no correlation between ratio Nrf2/Keap1 and catalase gene expression. In conclusion, administration of 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one can improve catalase gene expression in hyperlipidemic rats. However, there is no correlation between the ratio of Nrf2/Keap1 gene expression and the catalase gene expression.

ABSTRAK

Kondisi hiperlipidemia menghasilkan lebih banyak radikal bebas dalam bentuk anion superoksida yang menyebabkan stress oksidatif. Komponen fitokimia dapat menurunkan stres oksidatif. Penelitian ini bertujuan untuk mengetahui hubungan antara ratio Nrf2/Keap1 dan ekspresi gen katalase hepar tikus hiperlipidemia yang diberi 7-hidroksi-2-(4-hidroksi-3-metoksifenil)-kroman-4-on. Dua puluh empat tikus *Rattus norvegicus* umur 8 minggu dengan berat sekitar 200 g dibagi secara acak menjadi 6 kelompok yaitu Kelompok 1, tikus normal (N), Kelompok 2 tikus hiperlipidemia (HL), Kelompok 3 tikus hiperlipidemia yang diberi simvastatin (HL+SV), Kelompok 4-6 tikus hiperlipidemia yang diberi 7-hidroksi-2-(4-hidroksi-3-metoksifenil)-kroman-4-on dengan dosis 10 mg (HL+10), 30 mg (HL+30) dan 90 mg (HL+90) berturut-turut, secara oral. Pada akhir penelitian, hewan coba didekapitasi dan hepar tikus digunakan untuk menganalisis rasio Nrf2/Keap1 dan ekspresi gen katalase. Data ratio antar kelompok dianalisis menggunakan Kruskal Wallis. Uji korelasi antara ratio Nrf2/Keap1 dan ekspresi gen katalase dengan uji Sperman. Pemberian 7-hidroksi-2-(4-hidroksi-3-metoksifenil)-kroman-4-on dengan 3 peringkat dosis berbeda dapat meningkatkan ekspresi gen katalase. Tidak ada korelasi antara ratio Nrf2/Keap1 dan ekspresi gen katalase. Dapat disimpulkan bahwa pemberian senyawa 7-hidroksi-2-(4-hidroksi-3-metoksifenil)-kroman-4-on cenderung dapat memperbaiki ekspresi gen katalase pada tikus hiperlipidemia. Namun demikian tidak ada hubungan antara ratio ekspresi gen Nrf/2-Keap1 ekspresi gen katalase.

Keywords:
Keap1
Nrf2
catalase
hyperlipidemia
liver
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INTRODUCTION

Hyperlipidemia is one of the risk factors for degenerative diseases and metabolic syndrome. Several diseases related to metabolic syndrome reached the top ten list of the world's deadliest diseases during 2015,[1] including cardiovascular disease, stroke and diabetes mellitus.[1–4] Hyperlipidemia is a lipid metabolism disorder characterized by an increase in cholesterol, triglycerides or a combination of both in the blood. Hyperlipidemia is caused, among other reasons, by unhealthy lifestyles, lack of physical activity and high-fat diet.[5] A high-fat diet is linked to excessive free radicals, resulting in more superoxide anion radicals that are the cause of oxidative stress. Oxidative stress causes damage to cellular components such as carbohydrates, fats, proteins and DNA resulting in the formation of new free radicals.[6] Nuclear factor erythroid 2-related factor 2 (Nrf2) is an oxidative stress regulator present in the cytoplasm. It is inactivated and regulated by binding of Kelch-like ECH-associated protein 1 (Keap 1) for proteasomal degradation targets.[7–9] Under conditions of oxidative stress, Nrf2 becomes separated from the complex and translocated to the nucleus, to bind to antioxidant-responsive elements (ARE), and further induce gene expression of endogenous antioxidants such as superoxide dismutase, glutathione peroxidase and catalase.[10] Decreasing the ratio of Nrf2/Keap1 can cause oxidative stress through a decrease in endogenous antioxidant enzymes.[11] Catalase is an enzyme that can change hydrogen peroxide (H_2O_2) to become water and oxygen, so it is less harmful for body components.[12]

Efforts to suppress the rate of oxidative stress are done with antioxidants, both endogenous and exogenous. Under normal conditions, the body will form a balance between the formation of free radicals with endogenous antioxidants. Frequent free radical formation keeps the body from maintaining this balance, causing cellular damage that results in the pathological states of various diseases. Therefore, for optimum health benefits, it is necessary to add compounds that can increase the work of endogenous antioxidants.

Currently, many studies have demonstrated the role of phytochemical compounds such as flavonoids in reducing oxidative stress. Flavonoids can decrease oxidative stress by reacting to free radicals.[13] A flavonoid compound has been isolated from mahogany seed (Swietenia macrophylla king) by Mursiti[14] and was identified as 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one. The aim of this study was to investigate the correlations between the ratio of Nrf2/Keap1 and catalase gene expression in livers of hyperlipidemic rats given 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one.

MATERIALS AND METHODS

Animal

Twenty-four male rats (Rattus norvegicus), 8 weeks old, weigh average of 200 g were obtained from the Department of Food and Nutrition/PAU (Pangan dan Gizi), Universitas Gadjah Mada, Yogyakarta, Indonesia. The rats were housed in individual cages and acclimatized to the laboratory condition (22-25^{o}C) and 12 h daylight cycle for 7 days with free access to food and water during the experimental period. The standard diet was AIN 93 M consisting of (g/kg): Cornstarch (465.692), casein (140), dextrinized cornstarch (155), sucrose (100), soybean oil (40), alphacel (50), AIN-93-MMX (35), L-cysteine 1.8, AIN-93-VM (10), choline bitartrate (2.5) and ter-butylhydroquinone (0.008). The present study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Pubic Health

and Nursing, Universitas Gadjah Mada, Yogyakarta.

Experimental design

Twenty-four rats were divided into 6 groups i.e. Group 1 was normal rats (N), Group 2 was hyperlipidemia rats (HL), Group 3 was hyperlipidemia rats treated withsimvastatin0.18mg/200gbw(HL+SV), and Groups 4-6 were hyperlipidemic rats treated with 7--hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one doses 10; 30; or 90 mg/200 g BW day (HL+10, HL+30 or HL+90 mg/200 g BW), respectively. Hyperlipidemic rats were induced by standard AIN 93 with added 2% pure cholesterol. The compounds were administered orally by gavages for 4 weeks, after which all of the animals were euthanized.

Compound isolation

Compound of 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one is a class of flavonoids that derived from mahogany seeds (S. macrophylla King). This compound was isolated by Mursiti.[14] The 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one was isolated by liquid vacuum chromatography and gravity column chromatography. Elucidation of structural characteristics was carried out using IR, UV, and GC spectrometers. In this study the dose calculation based on our previous study.[15]

Gene expression with quantification PCR (q-PCR)

cDNA was synthesized using the iScript mix Biorad® kit according to the protocol of the producer. SsoFast™Evagreen® supermix Biorad® was used for q-PCR on a Biorad iCycler model CFX 96 Real-Time System. The primers used for cDNA amplification were forward 5'-AACCCCATGACCAACCAGTG- 3' and reverse 5'-CACTCGTCTCGATCTGGCTC-3' for kelch-like ECH-associated protein 1 (*Keap1*; 161 bp); forward 5'-ATTGCCGTCCGATTCTCC-3' and reverse 5'-CCAGTTACCATCTTCAGTGTAG-3' for Catalase; Forward 5'-GCCTTCCTCTGCTGCCATTAGTC-3' and reverse 5'GTGCCTTCAGTGTGCTTCTGGTT-3' for Nuclear factor erythroid related factor 2 (Nrf-2; 111 bp), forward 5'-GAATCTCTTCATTCTTGCCATT-3' and reverse 5'-GGCATAGAGGTCTTTACGGATG-3' for *beta actin* gene (240bp). The q-PCR reaction was conducted individually with each gene using the same internal control *beta actin* gene. The program for cDNA amplification was 5 min at 95 ^{o}C, followed by 40 cycles at 95 ^{o}C for 60 s, and 57 ^{o}C for 60 s.

Data analysis

All results were expressed as mean ± standard error of mean (SEM). The ratio of *Nrf2/Keap* 1 and *catalase* gene expression between the groups was analyzed by One Way ANOVA test. The correlations between *Nrf2/Keap 1* and *catalase* gene expression were analyzed by *Spearman* test. Data were considered statistically significant if p values were lower than 0.05.

RESULTS

As shown in FIGURE 1 the ratio of Nrf2/Keap 1 in rat livers after the administration of the 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one doses 30 mg/200 g BW was lower than the doses 10 and 90 mg/200 g BW ($p > 0.05$).

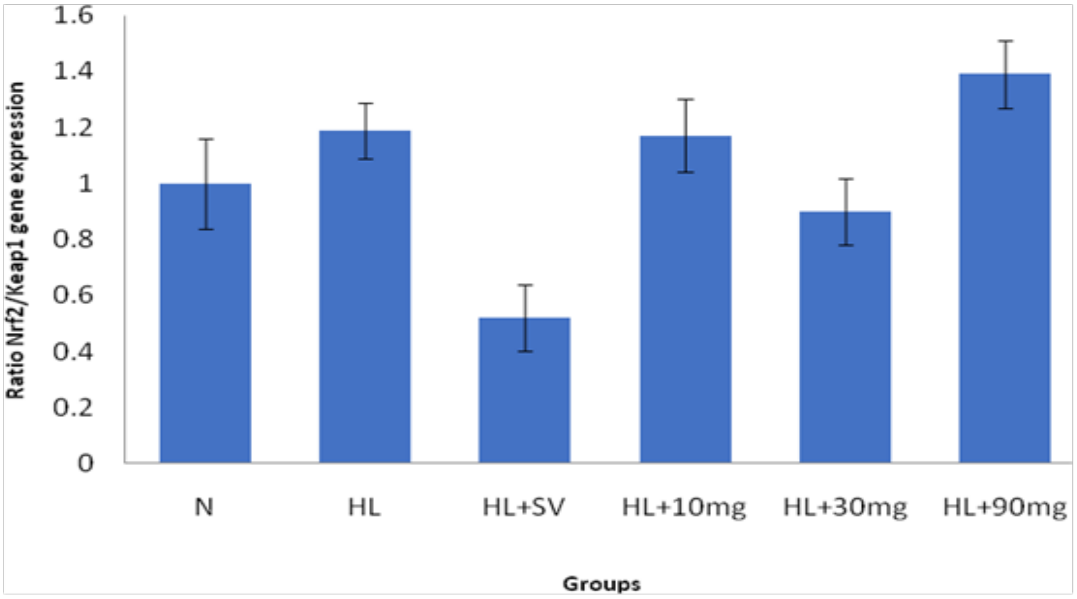

FIGURE 1. Ratio Nrf2/Keap1 gene expression. N, Normal rats; HL, hyperlipidemia rats; HL+SV, hyperlipidemia rats +.18mg/200 g BW simvastatin; HL+10, hyperlipidemia rats + 10mg/200 g BW Flavonoid; HL+ 30, hyperlipidemia rats + 30mg/200 g BW; Flavonoid and HL+90, hyperlipidemia rats + 90mg/200 g BW Flavonoid. N= 24 rats. Data were presented as mean ± SEM; p=0.400, according to one way Anova.

The relative expression of catalase in rat livers after administration of the 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one doses 10, 30 and 90 mg/200 g BW for four weeks were not significantly different (p >0.05) (FIGURE 2).

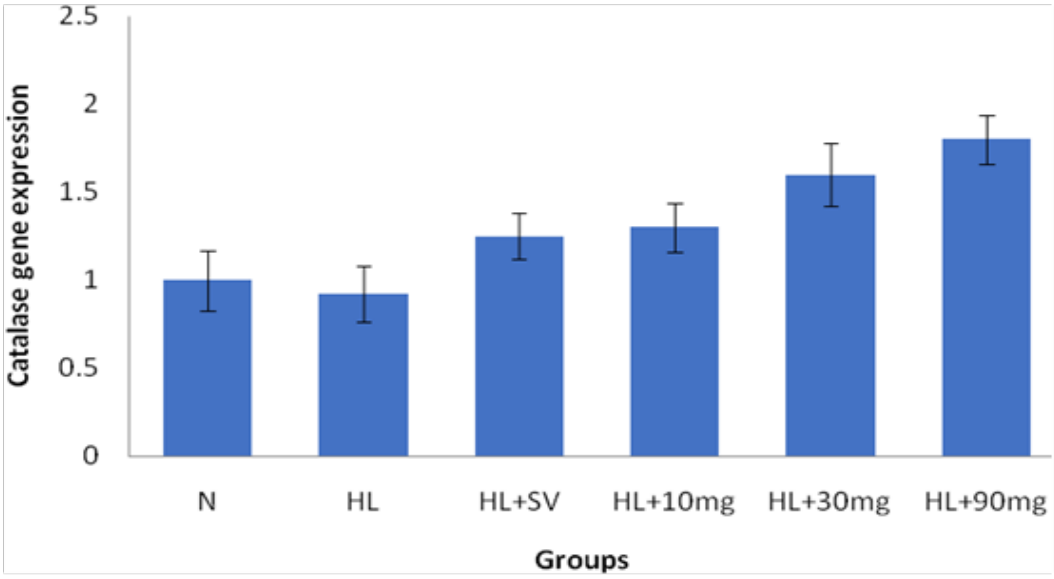

FIGURE 2. Catalase gene expression. N, Normal rats; HL, hyperlipidemia rats; HL+SV, hyperlipidemia rats + 0.18mg/200 g BW simvastatin; HL+10, hyperlipidemia rats + 10mg/200 g BW Flavonoid; HL+ 30, hyperlipidemia rats + 30mg/200 g BW; Flavonoid and HL+90, hyperlipidemia rats + 90mg/200 g BW Flavonoid. N= 24 rats. Data were presented as mean ± SEM; p=0.056, according to one way Anova.

Based on Spearman's analysis to determine correlations between ratio of Nrf2/Keap1 and catalase gene expression, the result showed that no correlation between both variables ($p > 0.05$) and ratio alteration does not change the catalase relative expression ($p > 0.05$).

DISCUSSION

Reactive oxygen species (ROS) are free radicals that can harm body components, such as lipid, DNA and protein. ROS are produced by normal metabolism in the body especially by mitochondria metabolism. One of ROS in the body is H_2O_2 that can be more radical if it reacts with other metal components to become a hydroxyl radical.[16] The body has antioxidant components to reduce increasing ROS by both non-enzymatic and enzymatic systems. In the non-enzymatic system, the body uses NADPH and GSH. In the enzymatic system, the body uses glutathione peroxidase, superoxide dismutase and catalase.[16,17] Catalase is an endogenous enzyme that can degrade H_2O_2 into water and oxygen. Its ability is very efficient in degrading peroxides but this enzyme needs iron or manganese as a cofactor.[12,18] Synthesis of catalase is regulated by Nrf2 as the transcription factor.[19]

Nuclear factor erythroid 2-related factor 2 is the most active transcriptional regulator in the leucine zipper transcription factor family. The biological activity Nrf2 is mainly exerted through an inhibitory control mechanism against Keap1.[20] Nuclear factor erythroid 2-related factor 2 is a key cytokine that regulates the expression of antioxidant substances *in vivo*. The normal expression of Nrf2 is critical for maintaining oxidant-antioxidant balance in the body.[21] The Keap1 protein contains a number of cysteine residues which are sensitive to ROS and modified by reactive electrophiles. Under normal conditions, Nrf2 is mainly present in the cytoplasm and binds to the Keap1 protein. Oxidation of Keap1 causes conformational changes that result in release of or inability to bind Nrf2 and direct it to proteasomal degradation. This condition of increased abundance of free Nrf2 protein within the cytosol is then translocated to the nucleus where it accumulates and binds to AREs which transcriptionally regulate expression of gene associated with antioxidant defense pathways. When the cells are exposed to a hyperoxic environment the conformation of Keap1 protein is changed, the binding to Nrf2 prevented. Nrf2 is then released into the nucleus, expressed extensively, and promotes the expression of downstream antioxidants, and resistance to oxidative damage.[22]

In this study, we determined the ratio of Nrf2- keap1 gene expression and catalase gene expression. The results showed that the highest ratio of Nrf2- keap1 was found in rats given 90mg/200gbw of 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one and the lowest was found in rats treated with 0.18mg/200 g BW of simvastatin. The ratio of Nrf2- keap1 was decreased following stimulation of oxidative stress induced by receptor activator of nuclear factor-κB ligand (RANKL). The flavonoid compounds such as catechin, and quercetin, will remove intracellular ROS[11], through modulation of the Nrf2/Keap1 pathway.[23] According to Habeos *et al.*[24] The administration of simvastatin did not affect the Nrf2 mRNA levels in rat livers. However, in our study we found the rats that received simvastatin had the lowest Nrf2 gene expression compared to all groups (data not shown).

In this study we also measured the correlations between Nrf2/Keap1 ratio and catalase gene expression. According to Kanzaki *et al.*,[11] Nrf2/Keap1 ratio has an association with cytoprotective enzyme expression. Decreasing the ratio Nrf2/Keap1 will be attenuating the cytoprotective enzyme expression.

However, our study showed that there was no correlation between Nrf2/Keap1 ratio and catalase gene expression.

CONCLUSION

In conclusion, administration of 7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-chromen-4-one compound can improve catalase gene expression in hyperlipidemic rats. However, there is no correlation between ratio of the Nrf2/Keap1 gene expression and the catalase gene expression.

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