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Catechins inhibit atherosclerosis in male rats on a high fat diet

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ABSTRACT

BACKGROUND
A catechin isolate from the green tea clone GMB 4, which shows antioxidant activity, may be a candidate drug for prevention of atherosclerosis. The aim of this study was to analyze the effect of catechin on endothelial nitric oxide synthase (eNOS) and p110 phosphoinositide 3-kinase inhibitor (PI3K) expression and on p38 mitogen activated protein kinase (MAPK) activity in male rats fed a high fat diet.

METHODS
Twenty five male Wistar rats were divided into the following five groups: rats on standard diet; rats on high fat diet; rats on high fat diet + catechin 3 mg/day; rats on high fat diet + catechin 6 mg/day; and rats on high fat diet + catechin 24 mg/day. eNOS and p110 PI3K expression and p38 MAPK activity were measured by means of ELISA.

RESULTS
High fat diet significantly increased eNOS expression, decreased p110 PI3K expression, and increased p38 MAPK activity in male rats, in comparison with standard diet ($p<0.05$). Administration of 3 mg/day catechin decreased eNOS expression compared to that in the high fat diet group without catechin ($p<0.05$). The administration of catechin increased p100 PI3K expression to a similar extent as that in the high fat diet groups with catechin 6 mg/day and 24 mg/day. Administration of catechin at all doses decreased p38 MAPK activity to the level of the standard diet group.

CONCLUSIONS
High fat diet increases eNOS expression, decreases PI3K expression, and increases p38 MAPK activity. Administration of catechin decreases eNOS expression, increases PI3K expression, and decreases p38 MAPK activity.

Keywords: Catechin, eNOS, p110 PI3K, p38 MAPK, high fat diet, male rats

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Katekin menghambat proses aterosklerosis pada tikus jantan dengan diet lemak tinggi

ABSTRAK

LATAR BELAKANG

Isolat golongan senyawa katekin dari teh hijau klon GMB 4, yang mempunyai aktivitas antioksidan, dapat dikembangkan sebagai kandidat preventif aterosklerosis. Penelitian ini bertujuan menilai efek katekin terhadap penghambatan penurunan ekspresi p110 phosphoinositide 3-kinase inhibitor *(PI3K) dan* endothelial nitric oxide synthase *(eNOS) serta penghambatan peningkatan aktivitas p38* mitogen activated protein kinase *(MAPK) pada tikus jantan dengan diet tinggi lemak.*

METODE

Dua puluh lima ekor tikus Wistar jantan yang terbagi dalam lima kelompok perlakuan, tikus dengan diet pakan standar; tikus dengan diet tinggi lemak; tikus dengan diet tinggi lemak + katekin 3 mg/hari; tikus dengan diet tinggi lemak + katekin 6 mg/hari; dan tikus dengan diet tinggi lemak + katekin 24 mg/hari. Ekspresi eNOS, p110 PI3K, dan aktivitas p38 MAPK dianalisis dengan teknik ELISA.

HASIL

Diet tinggi lemak meningkatkan ekspresi eNOS, menurunkan ekspresi p110 PI3K, meningkatkan aktivitas p38 MAPK secara bermakna dibandingkan kelompok diet standar ($p<0,05$). Pemberian katekin dosis 3 mg/hari meningkatkan ekspresi eNOS secara bermakna dibandingkan diet standar dan diet tinggi lemak ($p<0,005$). Pemberian katekin meningkatkan ekspresi p110 PI3K secara bermakna dibandingkan diet tinggi lemak ($p<0,05$), akan tetapi baru dapat mencapai ekspresi pada diet standar di dosis 6 mg/hari dan 24 mg/hari. Pemberian katekin berbagai dosis terbukti menurunkan aktivitas p38 MAPK secara bermakna dibandingkan diet tinggi lemak ($p<0,01$), bahkan mencapai aktivitas p38 MAPK diet standar.

KESIMPULAN

Diet tinggi lemak meningkatkan ekspresi eNOS, menurunkan ekspresi PI3K, dan meningkatan ekspresi p38 MAPK. Pemberian katekin dapat menurunkan ekspresi eNOS, meningkatkan ekspresi PI3K, dan menurunkan aktivitas p38 MAPK.

Kata kunci: *Isolat katekin, eNOS, p110 PI3K, p38 MAPK, diet tinggi lemak, tikus jantan*

INTRODUCTION

According to WHO estimates, annually 3.8 million males and 3.4 million females worldwide die from coronary heart disease. Atherosclerosis is the principal contributor to the pathomechanism of coronary heart disease.[1] One of the risk factors causing the progressivity of atherosclerosis is dyslipidemia resulting from a high fat diet and obesity. A high fat diet may trigger the development of chronic inflammation leading to endothelial dysfunction. The contribution of endothelial dysfunction to the pathogenesis of atherosclerosis is via uncoupling (deregulation) of endothelial nitric oxide synthase (eNOS). Uncoupling of eNOS triggers the formation of superoxide anions (O_2^-) and decreases NO release. Superoxide anions may react with NO to form peroxynitrites for inactivation of tetrahydro biopterin (BH_4), which in turn increases accumulation of asymmetric dimethylarginine (ADMA), the endogenous inhibitor of eNOS.[2] In addtion to causing

endothelial dysfunction, accumulation of toxic lipid metabolites, i.e. fatty acyl coenzyme A (CoA), diacylglycerol, and ceramides in tissues and arteries causes the development of insulin resistance.[3] The latter condition triggers abnormal insulin signal transduction in endothelial cells, involving two main pathways, i.e. the phosphoinositide 3-kinase inhibitor (PI3K) and mitogen activated protein kinase (MAPK) pathways.[4]

Insulin resistance decreases PI3K signaling and increases MAPK signaling. Continuous stimulation of MAPK activity causes vascular smooth muscle cell (VSMC) proliferation, increased collagen synthesis, overproduction of growth factors and pro-inflammatory cytokines, thereby accelerating the development of atherosclerosis.[3]

Among the naturally occurring substances that have antioxidant potential are the catechins isolated from the tea plant (*Camellia sinensis*). The Gambung Research Center for Tea and Quinine have developed green tea GMB4 clones with higher levels of catechins (14% - 16%) extracted from the third level leaf buds.[5] The number of hydroxyphenolic and galloyl groups and the epimerization structure of the isolates affect their activity despite similar levels of total catechins. There are several studies in support of the activity of tea catechins on vascular function, such as the study conducted by Anter et al. which shows that the polyphenol fraction of black tea stimulates eNOS catalytic activity. The mechanism involves stimulation-mediated activation of p38 MAPK and PI3K/Akt pathways leading to eNOS phosphorylation at serine 177 and dephosphorylation at Thr-495. This activity results in the activation of calmodulin-dependent eNOS and increased NO bioactivity.[6] Other studies have found strong evidence of NO involvement in the induction of vasorelaxation by tea polyphenols.[7] Catechins with their galloyl groups are natural inhibitors of tyrosine kinases that can modify the activity of various signaling kinases such as extra cellular signal-regulated kinase 1 and 2 (ERK1/2), protein kinase B (Akt), PI3K and p38 MAPK.[8] The hydroxyphenolic groups of catechins contribute free radical scavengers, inhibition of lipid peroxidation and hydrolysis of fat, while their galloyl groups contribute to the production of prostacyclins, reduction in vascular cell adhesion molecule-1 (VCAM-1) expression and inhibition of VSMC proliferation.[9]

On the basis of the results of abovementiomed studies, the aim of the present study was to analyze the effect of administration of a catechin isolate from the green tea clone GMB4 on the expression of eNOS and p110 PI3K, and on p38 MAPK activity in male Wistar rats on a high fat diet.

METHODS

Design of the study

A laboratory experimental study with controlled post test design, conducted from June 2010 to March 2011 in the Physiology Laboratory of the Faculty of Medicine, Brawijaya University, Malang.

Animals

In acccordance with the Federer formula, a total of 25 male Wistar rats *(Rattus norvegicus)* aged 7-8 weeks and weighing 130 - 155 grams, were divided into five intervention groups, which were fed the following diets: standard diet, high fat diet, high fat diet + catechin 3 mg/day, high fat diet + catechin 6 mg/day, and high fat diet + catechin 24 mg/day.[10] Before undergoing the interventions, the rats were adapted to laboratory conditions for 7 days. The interventions were conducted for 60 days, after which the rats were sacrificed to obtain their aortas.

Preparation of animal feed

The standard feed consisted of PARS chicken feed (containing water, protein, lipid, fiber, ash, calcium, phosphorus, antibiotics, and coccidiostatics, to a total content of 66.6%, and 33.4% wheat flour). The high fat diet was a combination of 57.3% standard feed (PARS) and

31.8% wheat flour), with the addition of cholesterol 1.9%, cholic acid 0.1% and pork fat 8.9%.[11]

Measurement of p110 PI3K and eNOS expression and p38 MAPK activity

The ELISA assay was performed as follows: after determination of the required number of microtiter wells, a standard curve was prepared, for which 100 ì l of assay buffer was pipetted into the blank well, and 100 ì l phosphorylated p38 MAPK or p110 PI3K standards 1-7 were pipetted into the designated treatment wells. Protein from aortic tissue samples isolated by means of radio immuno precipitation assay (RIPA) buffer was used for measurement of p38 MAPK and p110 PI3K concentrations. For the treatment samples, 100 ì l of treatment sample was pipetted into each well. The microtiter plates were incubated at 37^0C for 2 hours. Then the wells were washed with wash buffer 3 x 400 ì l. Next, each of the wells, with the exception of the blank, was filled with 100 ì l conjugate.

After further incubation of the microtiter plates at 37^0C for 30 minutes, the wells were each washed again with wash buffer 3 x 400 ì l. Subsequently the substrate, i.e. 100 ìl 3,3',5,5' -tetramethylbenzidine (TMB), was pipetted into each well, and the microtiter plates were again incubated for 30 minutes at room temperature. To stop the conjugation reaction, 100 µl HCl was pipetted into each well and left to react for 5 minutes. Absorbance readings were performed at an optical density (OD) of 492 nm.[12] For NOS and p110 PI3K expression, the measurements were in ìg/mL and pg/mL, respectively, while p38 MAPK activity was expressed in pg/mL.

Statistical analysis

Normally distributed data on p38 MAPK activity and p110 PI3K expression from each intervention are presented as mean and standard deviation. Non-normally distributed data are presented as the median, or as the mean and standard deviation after appropriate transformation. Data analysis was by means of one-way ANOVA followed by the Tukey test, using SPSS for Windows version 17.

Ethical clearance

This study was accorded ethical clearance by the Commission of Medical Research Ethics, Faculty of Medicine, Brawijaya University, Malang.

RESULTS

Based on the ANOVA results, there was a significant difference in aortic eNOS expression between intervention groups ($p<0.05$). From the results of the Tukey test it was concluded that high fat diet significantly increased eNOS expression in comparison with the standard diet group ($p<0.05$). Catechin administration at a dose of 3 mg/day significantly increased eNOS expression in comparison with the standard and high fat diets ($p<0.05$). Administration of catechin at a dose of 6 mg/day significantly reduced eNOS expression ($p<0.05$) in comparison with the high fat diet, although the reduction did not reach the level of the standard diet. Administration of catechin at a dose of 24 mg/day did not significantly reduce eNOS levels as compared with the high fat diet ($p>0.05$) (Table 1).

Table 1. Mean eNOS and p110 PI3K expression in the intervention groups

Expression	Standard diet	High fat diet + catechin isolate			
		0 mg/day	3 mg/day	6 mg/day	24 mg/day
eNOS (µg/mL)	1.21 ±0.14	2.18 ± 0.12^{a}	2.83 ± 0.18^{ab}	1.69 ± 0.15^{abc}	2.09 ± 0.61^{acd}
p110 PI3K (pg/mL)	38.69 ±0.41	32.87 ± 0.41^{a}	33.90 ± 0.20^{a}	38.29 ± 3.28^{bc}	41.27 ± 0.97^{bc}

Table 2. Mean p38 MAPK activity in the intervention groups

Activity	Standard diet	High fat diet + catechin isolate			
		0 mg/day	3 mg/day	6 mg/day	24 mg/day
p38 MAPK (pg/mL)	623.26 ± 35.16	1136.06 ± 295.71[a]	480.86 ± 132.49[b]	464.06 ± 103.75[b]	344.86 ± 23.22[b]

According to ANOVA, mean aortic p110 PI3K expression showed significant differences between intervention groups ($p<0.05$). From Tukey's test it was concluded that the high fat diet significantly decreased p110 PI3K expression, in comparison with the standard diet ($p<0.05$). Administration of catechins significantly increased p110 PI3K expression in comparison with the high fat diet ($p<0.05$), but reached the expression level of the standard diet only at the doses of 6 mg/day and 24 mg/day (Table 1).

As shown in Table 2, aortic p38 MAPK activities differed significantly between intervention groups ($p<0.05$). Tukey's test indicated that the high fat diet significantly increased p38 MAPK activity in comparison with the high fat diet ($p<0.05$). Administration of catechins at various doses was shown to effect an exceedingly significant reduction in p38 MAPK activity in comparison with the high fat diet ($p<0.01$), and even reached the p38 MAPK activity level of the standard diet.

DISCUSSION

Nitric oxide (NO) has vasodilator effects, prevents platelet adhesion, aggregation, and recruitment, inhibits migration and growth of smooth muscle, regulates interaction of platelets and blood vessels, and inhibits oxidation of low density lipoprotein.[13] In the present study, a high fat diet significantly triggered increased expression of eNOS, as compared with a standard diet. Increased eNOS expression is a compensatory mechanism to raised blood cholesterol concentrations,[13,14] and may be triggered by Akt-dependent calcium mobilizing or phosphorylating agents. In this study, a high fat diet was the stimulus for NO production via phosphorylation of eNOS on serine 177 through the PI3-kinase/Akt pathway.[15,16]

Administration of catechins at a dosage of 3 mg/day significantly increased eNOS expression as compared with that caused by the high fat diet. This indicates that this dose increases the stimulus for NO production via phosphorylation of eNOS on serine 177 through the PI3 kinase/Akt pathway, apart from the stimulus of the high fat diet. By contrast, in a previous study administration of feed containing catechins 0.3 % (w/w) for 10 days did not increase eNOS expression in aorta homogenates in comparison with controls.[17] These differing results were presumably due to differences in duration of catechin administration.

Administration of catechins at a dosage of 6 mg/day decreased eNOS expression as a result of the high fat diet, although not to the same extent as with a normal diet. Catechins may inhibit increased eNOS expression, because of their ability to inhibit the decrease in stimulus for increased NO requirement, by means of their antioxidant mechanism to prevent low density lipoprotein (LDL) oxidation, also known as their hypocholesterolemic effect. In addition, catechins also increase phosphorylation of eNOS and Akt.[18] Catechins even increase the bioavailability of NO through inhibition of NADPH oxidase.[19] The results of this study differ from those of Ou et al.,[20] where catechins did not trigger changes in eNOS expression. At a catechin dose above 6 mg/day eNOS was not reduced in comparison to that due to the high fat diet. This indicates that 6 mg/day was the effective dose of catechins for counteracting the

decreased aortic eNOS expression due to the high fat diet.

In the study of Ou et al.[20] on human umbilical vein endothelial cells (HUVEC) it was found that oxidized LDL activates oxidized low density lipoprotein (LOX-1) to trigger the formation of reactive oxygen compounds. Furthermore, reactive oxygen compounds block PI3K, leading to subsequent increase in eNOS expression. High fat diet significantly decreased p110 PI3K expression, in comparison with the group on standard diet. The decreased expression of PI3K in this study was caused by increased oxidized LDL that activated LOX-1 to trigger the formation of reactive oxygen compounds to block PI3K. Administration of catechins significantly increased the expression of p110 PI3K, as compared with the high fat diet ($p<0.05$), but could only equal PI3K expression on the standard diet at a dose of 6 mg/day and 24 mg/day. The increased PI3K expression was due to the antioxidant catechins inhibiting both oxidized LDL and scavengers of reactive oxygen compounds.

MAPK has implications on various physiological processes, among others cell proliferation, differentiation, and apoptosis. There are three main types of MAPKs in mammalian cells, viz. ERK1/2, JNK, and p38 MAPKs. Activation of MAPK modulates several steps in the inflammatory cascade.[21] The high fat diet significantly increased p38 MAPK activity in comparison with the standard diet. Increased p38 MAPK activity is due to increased oxidized LDL in endothelial cells or hepatocytes, as a result of the high fat diet.[20,22] Administration of catechins at various doses was shown to most significantly decrease aortic p38 MAPK activity in comparison with the high fat diet, and even equalled the activity level of p38 MAPK on standard diet. These findings are consistent with those of the study of Choi et al.,[23] who found that (-) epigallocatechin gallate inhibited phosphorylation of p38 MAPK in endothelial cells. The mechanism of catechin-induced inhibition of MAPK activity is based on the metal-chelating activity of catechins.[24] The activity of kinase receptors are dependent on divalent cations, which are removed through chelation by catechins, thus inactivating the receptors. In addition, the antioxidant activity of catechins is also capable of decreasing p38 MAPK activity.[20]

The administration of catechins to obese patients reduces insulin resistance, thus preventing progessivity of atherosclerosis. This may be caused by decreased expression of eNOS, which acts on the vasodilator NO, and by decreased p38 MAPK activity, which inhibits VSMC proliferation. One limitation of this study was that it did not investigate other signaling pathways apart from PI3K and MAPK, which play a role in the development of insulin resistance and atherosclerosis, respectively.

CONCLUSIONS

A high fat diet incrases eNOS expression, decreases PI3K expression, and increases p38 MAPK expression. Administration of catechins is capable of decreasing eNOS expression, increasing PI3K expression, and decreasing p38 MAPK activity.

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