ระดับเมแทบอไลต์ของไนตริกออกไซด์ในซีรัมของคนที่มีความผิดปกติของระดับไขมันในเลือด

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บทคัดย่อ

ไนตริกออกไซด์ (nitric oxide, NO) เป็นโมเลกุลส่งสัญญาณที่มีบทบาทสำคัญในการรักษาสมดุลของหลอดเลือด การสร้างไนตริกออกไซด์ลดลงในภาวะการสูญเสียการทำงานของเซลล์เอนโดธีเลียล ซึ่งเป็นขั้นตอนสำคัญในการเกิดภาวะหลอดเลือดแดงแข็งและโรคหัวใจและหลอดเลือด ความผิดปกติของระดับไขมันในเลือดเป็นปัจจัยเสี่ยงสำคัญของภาวะหลอดเลือดแดงแข็งและโรคหัวใจและหลอดเลือด ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์ในการวิเคราะห์ระดับเมแทบอไลต์ของไนตริกออกไซด์ในซีรัมของผู้ที่มีความผิดปกติของระดับไขมันในเลือดด้วยวิธีทางเคมีอย่างง่ายและราคาไม่สูง (Griess method) เพื่อประเมินการสูญเสียการทำงานของเซลล์เอนโดธีเลียล กลุ่มตัวอย่างคืออาสาสมัคร 200 คน ที่มีอายุ 35-60 ปี ไม่สูบบุหรี่ และไม่มีโรคประจำตัว รวมถึงโรคเบาหวาน และโรคความดันโลหิตสูง โดยแบ่งอาสาสมัครเป็น 2 กลุ่ม คือ กลุ่มที่มีความผิดปกติของระดับไขมันในเลือด (total cholesterol > 200 mg/dL, triglyceride > 150 mg/dL และ LDL cholesterol > 130 mg/dL) และกลุ่มที่มีระดับไขมันในเลือดปกติ ข้อมูลทั่วไปที่บันทึก ได้แก่ อายุ เพศ และดัชนีมวลกาย (body mass index, BMI) ผลการวิเคราะห์พบว่าระดับเมแทบอไลต์ของไนตริกออกไซด์ในซีรัมของผู้ที่มีความผิดปกติของระดับไขมันในเลือด (43.99 ± 9.34 μM) มีค่าต่ำกว่าในซีรัมของผู้ที่มีระดับไขมันในเลือดปกติ (46.03 ± 15.19 μM) เล็กน้อย แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ ระดับเมแทบอไลต์ของไนตริกออกไซด์ในซีรัม ยังไม่สัมพันธ์กับอายุ เพศ และดัชนีมวลกาย โดยสรุป ระดับเมแทบอไลต์ของไนตริกออกไซด์ในซีรัมไม่เปลี่ยนแปลงในผู้ที่มีความผิดปกติของระดับไขมันในเลือด เมื่อเทียบกับผู้ที่มีระดับไขมันในเลือดปกติ ดังนั้นระดับไนตริกออกไซด์ในซีรัมอาจไม่เหมาะสำหรับการประเมินภาวะการสูญเสียการทำงานของเซลล์เอนโดธีเลียล

คำสำคัญ: ความผิดปกติของระดับไขมันในเลือด การสูญเสียหน้าที่ของเซลล์เอนโดธีเลียล ไนตริกออกไซด์

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Serum Levels of Nitric Oxide Metabolites in Dyslipidemia Individuals

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Abstract

Nitric oxide (NO) is a signaling molecule with an important role in vascular homeostasis. Decreased production of NO is observed in individuals with endothelial dysfunction, which is an important step in the pathogenesis of atherosclerosis and cardiovascular disease (CVD). Dyslipidemia is considered as a risk factor of atherosclerosis and CVD. Therefore, the objective of this study was to determine NO metabolite levels in serum of dyslipidemia subjects by a simple chemical reaction, Griess method, in order to evaluate endothelial dysfunction. Two hundred subjects, 35-60 years old non-smokers with no history of diabetes and hypertension, were recruited and divided into two groups; dyslipidemia (total cholesterol > 200 mg/dL, triglyceride > 150 mg/dL and LDL cholesterol > 130 mg/dL) and normolipemia. General data, age, sex and body mass index (BMI), were collected. Serum NO metabolite levels were slightly lower in dyslipidemia subjects (43.99 ± 9.34 μM)) than in the normolipemia (46.03 ± 15.19 μM). However, they were not significantly different. In addition, serum levels of NO were not correlated with age, sex and BMI. In conclusion, NO metabolite levels did not change in dyslipidemia subjects, compared to normolipemia; therefore, it may not be appropriate for evaluating endothelial dysfunction.

Keywords: Dyslipidemia, Endothelial dysfunction, Nitric oxide

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Introduction

Nitric oxide (NO) is a signaling molecule in the vasculature, mainly produced from vascular endothelial cells.[1, 2] NO plays an important role in vascular homeostasis.[3] Its functions include vasodilation and platelet inhibition. Because of its short half-life (6-30 seconds), there is no storage of NO and its levels depend on levels of synthesis, catalyzed by nitric oxide synthase (NOS). There are 3 NOS isoforms; 1) NOS-1 (neuronal NOS, nNOS), expressed in nervous tissue and function in neurotransmission, 2) NOS-2 (inducible NOS, iNOS), expressed in immune and endothelial cells and activated by compounds, including cytokines and lipopolysaccharides (LPS) to mediate inflammation and 3) NOS-3 (endothelial NOS, eNOS), expressed in endothelial cells to maintain vascular integrity.[4]

Cardiovascular disease (CVD) is a major cause of death among non-communicable disease in Thailand. Dyslipidemia is a primary risk factor of CVD and associated with atherosclerosis and other risk factors of CVD, including insulin resistance and hypertension.[5, 6] Moreover, prevalence of dyslipidemia has been increasing in Thailand.[7] Pathogenesis of atherosclerosis and CVD involves endothelial dysfunction, which is an early marker of atherosclerosis.[6, 8, 9] Decreased NO levels have been shown as a marker of endothelial dysfunction, and its chemical analysis can be done by a simple Griess method.[10-12] Since it is less expensive than immunological assay used for other markers, analysis of NO is a good candidate marker to reveal endothelial dysfunction. The present study aimed to determine NO metabolites (NO_x) in dyslipidemia individuals in order to evaluate endothelial dysfunction.

Materials and methods

Sample collection

This research project was approved by the Ethical Review Sub-Committee Board for Human Research Involving Sciences, Thammasat University, No. 3 (project no. 131/2561). Two hundred subjects, who came for annual health check-up at the Public Health Service Center 27, Health Department, Bangkok, Thailand, were recruited into the study. They were 35-60 years old, non-smoker with no history of hypertension, diabetes or any other serious illness at the time of recruitment. The subjects were classified into two groups: dyslipidemia (n = 100) and normolipemia (n = 100). Dyslipidemia was determined by blood lipid results (total cholesterol >200 mg/dL, triglyceride >150 mg/dL and LDL cholesterol >130 mg/dL).

Biochemical analysis

Blood collection was done after 12 hours of fasting and serum was prepared. Blood lipid profile (total cholesterol, triglyceride and HDL-cholesterol, (HDL-C) was analysed by using FUJIFILM FDC NX500 dry chemistry

automate (FUJIFILM Europe GmbH Medical Systems Division, Germany). LDL-cholesterol (LDL-C) was calculated using the Friedewald equation. After lipid analysis, serum samples were kept at -70°C until the analysis of nitric oxide.

Analysis of nitric oxide

Nitric oxide was analyzed as its metabolites (nitrate and nitrite, NO_x) by using Griess assay.[10, 11] The principle is based on the reaction of nitrite and sulfanilamide, forming diazonium salt, which is then reacted with N-(1-Naphthyl) ethylenediamine dihydrochloride. As a result, an azo compound is formed and monitored by spectrophotometric measurement at 540 nm. First, serum was deproteinated by using acetonitrile. After the centrifugation at 10,000xg for 15 minutes, the deproteinated supernatant was then used for the analysis. Griess reagents consisted of 50 µL sulfanilamide (1%), 50 µL N- (1-Naphthyl) ethylenediamine dihydrochloride (0.2%) and 100 µL vanadium chloride (III) (0.5%). Nitrate standard (0-100 µM) or supernatants (100 µL) were mixed with Griess reagents and incubated at 60°C for 15 minutes. Then the absorbance at 540 nm were measured. The NO_x levels were calculated against linear standard curve. Precision and accuracy of the method were determined (CV = 3.95% and recovery = 101.87%).

Statistical analysis

Descriptive statistics is used for demographic variables (sex, age and BMI) and biochemical laboratory results (lipid profile and NO_x). The laboratory results were shown as mean ± SD. The difference of general characteristics between two groups was tested by using the Chi-square test. Student's *t*-test was used for comparison of means between two groups and correlations were tested by using Pearson's correlation.The *p-value* less than 0.05 was considered as statistically significance. The SPSS software version 12 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Results

Subjects were classified into two groups, dyslipidemia and normolipemia. Dyslipidemia subjects had total cholesterol > 200 mg/dL, triglyceride >150 mg/dL and LDL-C >130 mg/dL, while normolipemia had total cholesterol < 200 mg/dL, triglyceride <150 mg/dL and LDL-C < 130 mg/dL. General data of the subjects are shown in Table 1. The average ages of dyslipidemia and normolipemia subjects were 51.33 ± 6.70 and 50.94 ± 6.70 years, respectively. The female to male ratio were 55:45 and 66:34 in dyslipidemia and normolipemia, respectively. There were no statistically significant differences in age, sex and BMI between dyslipidemia and

Table 1 Age, sex and BMI of dyslipidemia and normolipemia subjects

Variable (unit)	Normolipemia	Dyslipidemia
	n = 100	n = 100
Age (year)	51.33 ± 6.70	50.94 ± 6.70
Female to male ratio	66.0:34.0	55.0:45.0
BMI (kg/m^2)	22.22 ± 2.08	21.74 ± 2.23
NO (μM)	46.03 ± 15.19	43.99 ± 9.34

Data are presented as mean ± SD.

normolipemia groups. All the subjects had normotensive blood pressure and normal fasting blood glucose. Blood lipid profiles are shown in Fig. 1. Dyslipidemia had significantly higher levels of total cholesterol, triglyceride and LDL-C (241.62 ± 28.09, 209.24 ± 58.33 and 168.68 ± 31.96 mg/dL, respectively) than those in normolipemia (174.57 ± 16.26, 81.64 ± 27.05 and 105.47 ± 16.65 mg/dL, respectively). In contrast, HDL

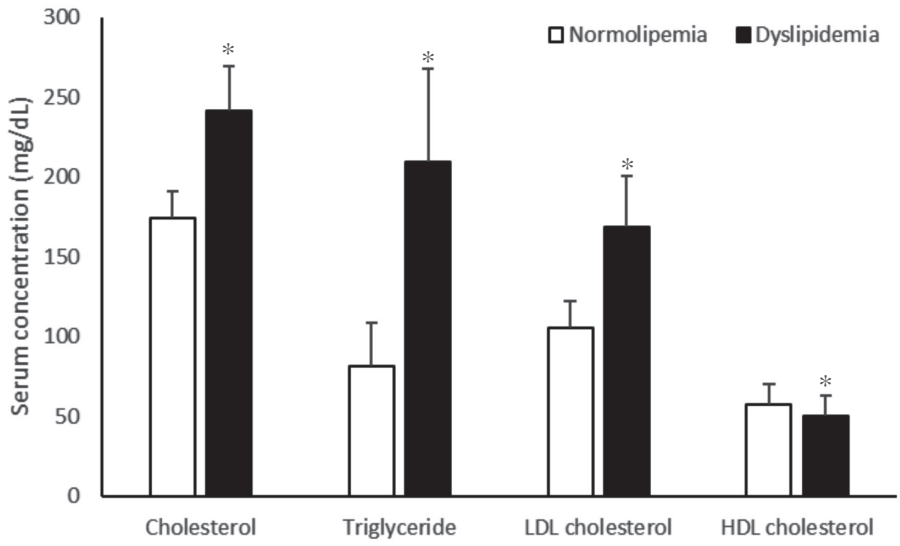

Fig. 1 Blood lipid profile of dyslipidemia and normolipemia subjects. Total cholesterol, triglyceride, LDL (LDL-C) and HDL (HDL-C) cholesterol were determined in serum of dyslipidemia (black bar, n = 100) and normolipemia (white bar, n = 100). Data are shown as mean ± SD. *; $p < 0.05$ when compared with normolipemia

cholesterol was lower in dyslipidemia (50.38 ± 12.88) than that in normolipemia (57.67 ± 12.19 mg/dL). Correlations among blood lipid profiles were tested and shown by correlation coefficient; r (Table 2). It was found that positive correlations among total cholesterol, triglyceride and LDL-C were significant at moderate to strong levels. However, a negative correlation of HDL-C to total cholesterol, triglyceride and LDL-C was observed at lower levels.

Serum levels of NO were shown as NO_x levels. Dyslipidemia subjects had slightly lower NO_x levels (43.99 ± 9.34 μM) than those from normolipemia subjects (46.03 ± 15.19 μM). Nevertheless, they were not significantly different. Then, normolipemia and dyslipidemia groups were subdivided by age, sex and BMI for further analysis. When categorized by age, there were no differences in NO_x levels in different age groups (Fig. 2A). Female and male subjects with either normolipemia or dyslipidemia had similar levels of NO_x (Fig. 2B). In addition, dyslipidemia subjects who were underweight (<18.5 kg/m^2), normal weight (18.5-22.9 kg/m^2), overweight (23.0-24.9 kg/m^2) and obese (25.0-29.9 kg/m^2), had similar levels of NO_x, compared with normolipemia subjects (Fig. 2C). When correlation tests were performed between NO_x and blood lipid profile, it was found that NO_x levels were not significantly correlated to age, sex, BMI, total cholesterol, triglyceride, LDL-C and HDL-C (Table 2).

Table 2 Correlations between nitric oxide and blood lipid profile (total cholesterol, triglyceride, LDL-C and HDL-C)

	Correlation coefficient (*p* value)				
	Nitric oxide	**Total C**	**Triglyceride**	**LDL-C**	**HDL-C**
Nitric oxide	1				
Total C	0.027 (0.703)	1			
Triglyceride	0.013 (0.859)	0.693 (0.000)**	1		
LDL-C	0.048 (0.503)	0.915 (0.000) **	0.601 (0.000)**	1	
HDL-C	0.013 (0.859)	-0.163 (0.021)*	-0.392 (0.000)**	-0.301 (0.000)**	1

**, significant levels were at $p < 0.01$; *, significant levels at $p < 0.05$; Correlations were tested by using Pearson's correlation.

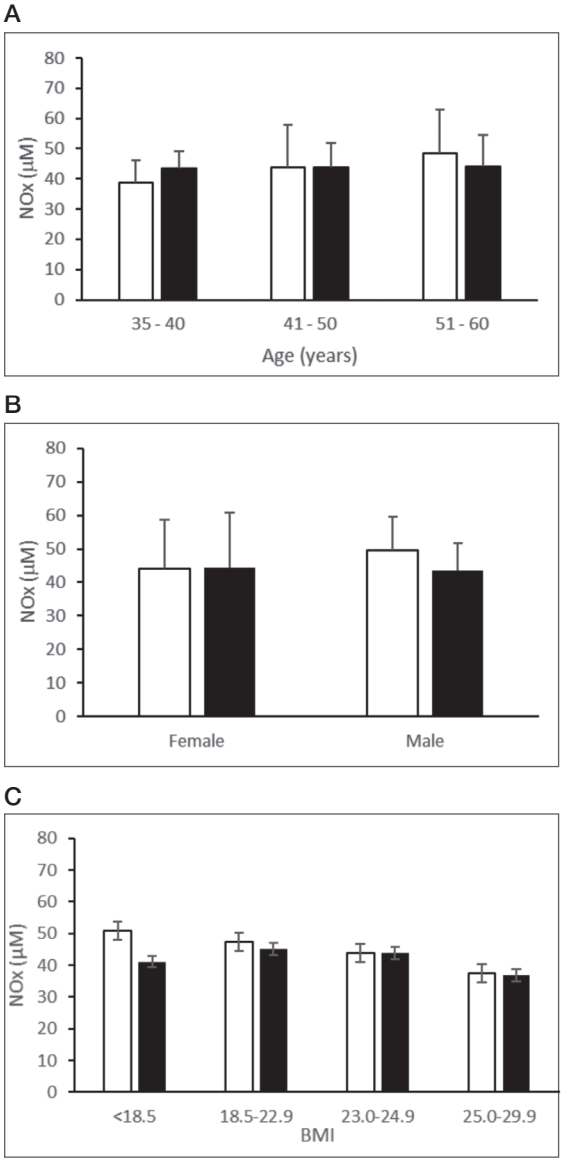

Fig. 2 Serum levels of nitric oxide (NO_x) in normolipemia and dyslipidemia, categorized by age (A), sex (B) and BMI (C). Age subgroups were 35-40, 41-50 and 51-60 years old (n = 10, 32 and 58 for normolipemia; n = 10, 33 and 57 for dyslipidemia group, respectively). Female and male ratio were 66:34 and 55:45 in normolipemia and dyslipidemia groups, respectively. BMI subgroups were <18.5, 18.5-22.9, 23.0-24.9 and 25.0-29.9 kg/m^2 (n = 8, 57, 31 and 5 for normolipemia; n = 10, 59, 30 and 1 for dyslipidemia group, respectively). NO_x levels in dyslipidemia are shown in black bar and normolipemia in white bar. Data are shown as mean ± SD. The number of age subgroups

Discussion

In the present study, dyslipidemia and normolipemia subjects were studied. It was observed that total cholesterol, triglyceride, LDL-C and HDL-C were significantly correlated. As previously shown, there are correlations among blood lipid profile.[13] Total cholesterol, triglyceride and LDL-C were related at moderate to high level, while HDL-C was associated with total cholesterol, triglyceride and LDL-C at lower level. Moreover, the levels of triglyceride in the dyslipidemia group were over two folds that of the normolipemia group. Triglyceride has been shown as an independent risk factor of CVD since triglyceride-rich lipoprotein can penetrate the endothelium and be phagocytosed by macrophage, contributing to plaque development.[14, 15] Therefore, according to the published guidelines, management of hypertriglyceridemia is recommended, including lifestyle intervention and triglyceride level-lowering medication.[16]

Moreover, NO_x levels in dyslipidemia subjects were slightly lower than those in normolipemia subjects (43.99 ± 9.34 VS 46.03 ± 15.19 μM). However, no significant differences were observed. Previous reports showed decreased NO levels in diabetes, hypertension, obesity and metabolic syndrome.[17-19] The levels were correlated with endothelial dysfunction, oxidative stress and inflammation.[20] Even though NO is primarily produced from endothelial cells, microbial products and proinflammatory cytokines also induce production of NO from immune cells.[21] In this study, subjects were recruited from patients who came for annual health check-up and were presumably healthy. However, it is possible that NO is induced in immune cells; and therefore hinders reduction of NO synthesis by endothelial cells. Indeed, previous studies have shown inconsistency in some conditions.[22, 23] It has been explained that this can be compensatory process to the loss of NO by dysfunctional endothelial cells. In addition, another study showed that rheumatoid arthritis, whose pathogenesis involves inflammation and endothelial dysfunction, has elevated levels of NO metabolite and there is an association between NO metabolite, inflammation and endothelial dysfunction.[24] This may explain why the reduction of NO metabolites was not seen in the present study.

Moreover, it has been shown that serum NO metabolites increase by age and men have higher NO levels than women.[25, 26] In addition, serum NO metabolites are elevated when BMI increases.[27] However, serum levels of NO metabolites were not different among age, sex and BMI subgroups in the present study. This agrees with a previous study showing that age, sex and BMI have no correlation with dyslipidemia.[28] It is possible that there are other factors affecting plasma NO levels and causing the conflicting results.

This study showed that NO_x levels did not have correlations with studied variables,

inconsistent with our hypothesis. The explanation of the results could be the effects of confounding factors. It was shown that diet and exercise affect plasma levels of NO metabolites.[29] Consequently, these two factors may affect plasma NO metabolites and the interferences mask the correlations between NO_x levels and endothelial dysfunction observed in dyslipidemia. In addition, the sample size in our study may be too small to represent the population.

In conclusion, our results reveal that serum NO_x levels had no association with age, sex, BMI, total cholesterol, triglyceride, LDL-C and HDL-C. Therefore, assessment of NO may not reflect endothelial dysfunction in dyslipidemia. Further study should be done in larger sample size with exploration of confounding factors and other markers of endothelial dysfunction.

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