Increased plasma glutamate in non-smokers with vasospastic angina pectoris is associated with plasma cystine and antioxidant capacity

Minako Oda, Kousuke Fujibayashi, Minoru Wakasa, Shintaro Takano, Wataru Fujita, Michihiko Kitayama, Hiroaki Nakanishi, Kazuyuki Saito, Yasuyuki Kawai and Kouji Kajinami

ABSTRACT

Objectives. Endothelial dysfunction caused by oxidative stress plays an important role in the development of vasospastic angina pectoris (VSAP). Glutamate causes endothelial dysfunction by generating oxidative stress, and it inhibits cystine import into endothelial cells via the cystine/glutamate antiporter (X_{C}^{-}) which leads to depletion of antioxidant glutathione. However, whether glutamate and cystine levels are implicated in the pathogenesis of VSAP remains unclear. We investigated plasma glutamate and cystine levels, oxidative stress markers and antioxidant capacity in non-smoker patients with VSAP to determine whether glutamate and cystine are associated with the development of VSAP. We assessed 49 non-smokers assigned to groups with (n = 27) and without (n = 22) VSAP, and also measured plasma glutamate, cystine, nitrotyrosine, reactive oxygen metabolites and biological antioxidant potential.

Results. Plasma glutamate and cystine values were significantly higher in the group with, than without VSAP (59.8 ± 25.7 vs. 43.5 ± 18.7 μmol/L, p = 0.016 and 35.3 ± 14.2 vs. 25.2 ± 9.1 μmol/L, p = 0.0056, respectively). Plasma glutamate and cystine values were significantly and positively associated (r = 0.32, p = 0.027). Levels of the oxidative stress markers nitrotyrosine and reactive oxygen metabolites, and biological antioxidant potential of as a measure of antioxidant capacity, did not significantly differ between the two groups. However, glutamate and biological antioxidant potential values were significantly and negatively associated (r = −0.3, p = 0.036). Conclusion. Plasma glutamate levels were increased in patients with VSAP who did not smoke, and they were positively associated with plasma cystine and negatively associated with the biological antioxidant potential levels.

Introduction

Coronary vasospasm is involved in the pathophysiology of variant angina, acute myocardial infarction and sudden cardiac death [1–5]. Endothelial dysfunction of coronary arteries caused by the overproduction of oxidative stress plays an important role in the pathogenesis of coronary spasm [6–8]. Under ischemic conditions, glutamate triggers the production of nitric oxide (NO) and superoxide leading to peroxynitrite formation via N-methyl-D-aspartate receptor activation in endothelial cells [9]. We previously found that levels of circulating nitrotyrosine, an oxidative stress marker produced via peroxynitrite modification of protein tyrosine residues, were increased after coronary vasospasm [10].

In addition to oxidative stress, decreased antioxidant capacity may be responsible for endothelial dysfunction. Glutathione is an important antioxidant that attenuates coronary vasospasm in patients with vasospastic angina pectoris (VSAP) [11] and reverses endothelial dysfunction in patients with atherosclerosis [12]. The synthesis of glutathione depends on the availability of the amino acid precursors, glutamate, glycine and cysteine [13]. Cystine is an oxidative form comprising two cysteines that are taken up by a specific cystine/glutamate antiporter system (X_{C}^{-}) in association with glutamate export. Extracellular glutamate competitively inhibits cystine import into endothelial cells [14–16]. Therefore, extracellular glutamate and cystine concentrations are crucial for glutathione biosynthesis.

Smoking induces powerful reactive oxygen species (ROS) throughout the body that comprise major risk factors for VSAP [17], and smoking might mask the effects of glutamate and cystine on endothelial dysfunction. Therefore, this study aimed to determine by measuring levels of plasma glutamate and cystine, oxidative stress markers and antioxidant capacity in patients with VSAP who showed only epicardial coronary artery spasm and were non-smokers.

Methods

Patients

Ninety consecutive Japanese patients with chest pain at rest and suspected VSAP were admitted to our hospital and...
assessed by diagnostic coronary angiography (CAG). We excluded six patients with CAG findings of ≥50% organic stenosis and 35 current and ex-smokers. Therefore, the remaining 49 patients who did not have significant organic stenosis (lumen diameter, <50%) underwent intracoronary acetylcholine provocation tests.

The Human Ethics Committee of Kanazawa Medical University approved the study protocol, which conformed to the ethical principles established in the Declaration of Helsinki. All included patients provided written informed consent to participate in the study.

**Catheterization and coronary angiography**

At least 24 h before the provocation test, nitrates, calcium channel blockers, and other anti-anginal drugs (except sublingual nitroglycerin) were withdrawn from all patients after admission. All CAG assessments proceeded in the morning after heparin (2000 U) was administered via the femoral approach using the standard Judkins technique. Acetylcholine for the provocation test was injected through a catheter into the right (20 or 50 μg) and left (50 or 100 μg) coronary arteries within 1 min. Angiographic assessment then proceeded 3 min from the start of each injection. We assessed ST-segment shifts on continuous recordings obtained using standard 12-lead electrocardiography. Patients informed the examiner if chest pain developed during provocation tests. We immediately started CAG when angina developed with chest pain and/or ST-segment shift, and 0.25 mg of nitroglycerin was injected into the responsible coronary artery to relieve the symptoms. Coronary vasospasm was defined as transient total (100%) or near-total (≥90%) occlusion of the provoked coronary artery that was reversible with nitroglycerin. Patients were considered VSAP positive if chest pain and angina developed with an ST-segment shift, and if coronary vasospasm was evident on angiograms. Among the 49 patients who underwent provocation tests, 27 and 22 were positive (VSAP-positive), and negative (VSAP-negative), respectively for vasospasm and cation tests, 27 and 22 were positive (VSAP-positive), and negative (VSAP-negative), respectively for vasospasm and the latter served as a control. After provocation, 0.25 mg of nitroglycerin was injected into both coronary arteries of VSAP-negative patients and into the non-responsible coronary artery of VSAP-positive patients to obtain maximal coronary dilation for final CAG image acquisition.

**Biological coronary risk factors, glutamate, cystine and 12 other amino acids in blood**

Leucine, isoleucine, valine, arginine, proline, glycine, citrulline and ornithine are associated with generating oxidative stress, whereas glutamine, cysteine, methionine and serine are associated with glutamate and cystine biosynthesis [18,19]. Serum and plasma samples were separated by centrifugation from blood collected from the femoral sheath before CAG and stored at −80°C. Samples for reactive oxygen metabolites and biological antioxidant potential tests were stored at 4°C for a maximum of 3 h. Samples for glutamate and cystine, and the other 12 amino acids were collected in EDTA-containing vacutainer tubes (Terumo, Tokyo, Japan). Plasma was obtained by centrifugation at 4°C and stored at −80°C until measurement. For rapid chromatographic separation and high sensitivity in the mass spectrometry, 3-aminohippuridyl-N-hydroxysuccinimidyl carbonate (APDS) (Fujifilm Wako Pure Chemical Industries Ltd, Osaka, Japan) was used as the derivatization reagent followed by liquid chromatography and mass spectrometry (LCMS-8030 Plus, Shimazu, Tokyo, Japan) [20]. Serum creatinine, plasma glucose, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein and triglyceride levels were measured using an automated biochemical analyzer (Hitachi, Tokyo, Japan). Serum concentrations of hemoglobin A1C (HbA1C) were determined by high-performance liquid chromatography using an automatic HbA1C analyzer (Tosoh, Tokyo, Japan).

**Measurement of nitrotyrosine in serum**

We measured nitrotyrosine using time-resolved fluorimunoassays that are 100-fold more sensitive than conventional enzyme-linked immunosorbent assays [21–23]. Fluorescence intensity was measured at 340 nm excitation and 615 nm emission, using an Arvo SX multilabel counter (Perkin-Elmer Life Sciences, Boston, MA, USA).

**Serum reactive oxygen metabolites and biological antioxidant potential**

We measured reactive oxygen metabolites and biological antioxidant potential in fresh serum within 3 h of blood collection using the Free Radical Analytical System 4 (Wismerll Co. Ltd, Tokyo, Japan). The reactive oxygen metabolites test detects hydroperoxides released from proteins in acidic medium that are converted to alkoxy and peroxyl radicals in the presence of transition metals that can oxidize alkylsubstituted aromatic amine (N,N-diethyli paraphenylene diamine) with increased absorbance at 505 nm. The method photometrically detects the density of colored complexes. One unit of reactive oxygen metabolites (U.CARR) corresponds to the amount of hydroperoxide that can be converted by superoxide dismutase to approximately 0.8 mg/dL H2O2 [24,25]. The biological antioxidant potential test to assess antioxidant activity of serum is based on the ability of a colored solution containing a source of ferric (Fe3+) ions to decolor when Fe3+ ions are reduced to ferrous ions (Fe2+). To assess the intensity of decoloration, the amount of reduced ferric ions was evaluated by photometrically as the antioxidant potential

**Statistical analyses**

Continuous variables are expressed as means ± standard deviation (SD). Values were compared between groups using Student t tests. Categorical variables of the patient’s clinical background were compared using χ2 tests. Relationships between two continuous variables were determined using
Spearman rank correlation coefficient. To examine the relationship between VSAP presentation and demographic and laboratory variables, including glutamate and cystine levels, a univariate logistic regression analysis was initially performed, followed by a stepwise multivariate logistic regression analysis. Age, gender (male = 1, female = 2), body mass index (BMI), creatinine and glutamate or cystine levels for multivariate logistic regression analysis model 1 or model 2, respectively, were used as initial independent variables. In this analysis, a stepwise backward selection was performed from a set of these variables by removing predictors based on Akaike Information Criteria (AIC) [27]. Data analyses were conducted by using Statflex software, version 6.0 (Artech Co., Ltd., Japan). All statistical tests were two-sided, and p values of <.05 were considered statistically significant.

Results

Clinical characteristics of the patients

Table 1 shows the clinical characteristics of the VSAP-positive and -negative patients. Mean age was more advanced in the VSAP-positive than VSPA-negative group, but the difference did not reach statistical significance. None of sex, BMI, prevalence of the coronary risk factors of hypertension, hyperlipidemia and diabetes mellitus or HbA1c and lipid profiles significantly differed between the groups. In contrast, creatinine values were significantly higher in VSAP-positive, than VSAP-negative patients. None of the VSAP-negative patients had significant narrowing of an involved coronary artery, electrocardiographic changes or chest pain during the procedure of the provocation test. None of the patients developed a ventricular rhythm disturbance, refractory spasm, myocardial infarction or other major complications.

Plasma glutamate, cystine and other 12 amino acids

Figure 1 shows that plasma glutamate and cystine values were significantly higher in the VSAP-positive (A) than VSAP-negative (B) patients, respectively. (59.8 ± 25.7 vs. 43.5 ± 18.7 μmol/L, p = .016 and 35.3 ± 14.2 vs. 25.2 ± 9.1 μmol/L, p = .0056, respectively). Table 2 shows values for the eight amino acids associated with oxidative production in endothelial cells and four amino acids associated with glutamate and cystine biosynthesis. Among all the measured amino acids, plasma cysteine was excluded from the table as it was barely detectable, probably due to extracellular oxidization to cystine [14].

Serum nitrotyrosine, reactive oxygen metabolites and biological antioxidant potential levels of the study group

We investigated oxidative stress markers to evaluate whether increased glutamate produces excess oxidative stress. Values for nitrotyrosine and reactive oxygen metabolites between the VASP-positive and -negative patients did not significantly differ (10.5 ± 5.2 vs. 12.4 ± 7.2 mg/mL, p = .28 and 362.1 ± 84.5 vs. 352.6 ± 67.2 U.CARR, p = .67, respectively;
Furthermore, glutamate was not associated with nitrotyrosine or reactive oxygen metabolites (data not shown), suggesting that less oxidative stress was produced in non-smokers with VSAP regardless of high plasma glutamate values. Levels of biological antioxidant potential as an indicator of antioxidant capacity also did not significantly differ between the VSAP-positive and -negative patients (2570.8 ± 506.3 vs. 2623.9 ± 653.2 µmol/L, p = .75; Figure 2(C)). However, plasma glutamate and biological antioxidant potential values were significantly and negatively associated (r = −0.30, p = .036; Figure 3(A)), suggesting that high levels of glutamate causes antioxidant capacity to decrease. Glutamate inhibits cystine uptake into the X̂C−, and we found significant association between them (r = 0.32, p = .027; Figure 3(B)), suggesting that the cystine elevation is a result of inhibited uptake into the X̂C− via increased glutamate-induced glutathione depletion and a subsequently low antioxidant capacity. Finally, we analyzed two models in multivariate logistic regression analyses that included age, gender, BMI and creatinine to determine independent determinants of VSAP. Model 1 included these factors and glutamate, but not cystine, and BMI was removed from these variables by a stepwise backward selection based on AIC. Model 2 included these factors and cystine, but not glutamate, and age, BMI and creatinine were removed from these variables in the same way as Model 1. The only independent determinants of VSAP in both models were glutamate and cystine. Age, sex, BMI and creatinine values were not significantly associated with VSAP (Table 3).

**Table 2.** Amino acids levels in both groups.

| Amino Acid | VSAP-positive | VSAP-negative | p   |
|------------|---------------|---------------|-----|
| Leucine (µmol/L) | 115.1 ± 19.6  | 113.4 ± 20.9  | .77 |
| Isoleucine (µmol/L) | 61.4 ± 13.7  | 60.3 ± 12.5  | .77 |
| Valine (µmol/L) | 196.5 ± 48.1  | 203.5 ± 32.3  | .56 |
| Arginine (µmol/L) | 84.8 ± 20.8  | 87.2 ± 15.9  | .66 |
| Proline (µmol/L) | 133.0 ± 37.4  | 137.1 ± 45.8  | .73 |
| Glycine (µmol/L) | 204.9 ± 53.0  | 214.3 ± 50.4  | .53 |
| Citrulline (µmol/L) | 30.4 ± 10.1  | 29.2 ± 6.5  | .64 |
| Ornithine (µmol/L) | 52.1 ± 14.4  | 47.7 ± 10.1  | .23 |
| Glutamine (µmol/L) | 525.4 ± 115.0 | 572.9 ± 72.2  | .10 |
| Methionine (µmol/L) | 22.0 ± 4.0  | 23.1 ± 4.8  | .39 |
| Serine (µmol/L) | 103.7 ± 19.7  | 102.2 ± 24.8  | .81 |

Data are presented as mean ± standard deviation.
Leucine, isoleucine, valine, arginine, proline, glycine, citrulline and ornithine are associated with endothelial dysfunction. Glutamine is a glutamate precursor. Serine and methionine are cysteine precursors.

**Discussion**

This clinical study showed that elevated plasma glutamate was associated with elevated cystine and decreased antioxidant capacity without elevated oxidative stress in patients with VSAP who do not smoke. This clinical evidence supports the notion that these two amino acids contribute to the pathogenesis of VSAP and if so, they might serve as novel risk factors for VSAP. Our results also suggested that an impaired antioxidant system is more important to the pathogenesis of VSAP than the excessive oxidative stress.

Endothelial dysfunction due to oxidative stress produced by several risk factors in the endothelial cells contributes to the progression of VSAP [6–10]. However, many of the conventional risk factors for atherosclerosis such as hypertension and diabetes are not apparently applicable to VSAP. Smoking is among the risk factors associated with VSAP and oxidative stress [11,17]. Therefore, our study design excluded patients who smoked to explore novel risk factors for VSAP.

Glutamate causes endothelial dysfunction by producing oxidative stress in the endothelial cells [9]. NO is normally generated after glutamate binds to N-methyl-D-aspartate receptor on endothelial cells, which leads to vasodilation [28,29]. Once ROS are generated by transient ischemia due to coronary spasm, peroxynitrite generated from ROS and NO induced by glutamate can oxidize many biomolecules, leading to endothelial dysfunction [30–32]. We measured nitrotyrosine levels because peroxynitrite production can be indirectly inferred by the presence of nitrotyrosine [33]. However, we could not prove that nitrotyrosine and reactive oxygen metabolites are elevated in patients with VSAP who do not smoke, suggesting that oxidative stress is less involved in the pathogenesis of VSAP among non-smokers.

In contrast, an antioxidant system involving glutathione protects vascular cells and other tissues against oxidative stress [34]. Human umbilical endothelial cells depleted of glutathione have increased susceptibility to oxidant damage [35]. Cystine is believed to be a rate-limiting precursor of...
glutathione, which is synthesized through the sequential enzymatic actions of glutamate cysteine ligase and glutathione synthetase. Systemic Xc⁻ is the sodium-independent antiporter responsible for cystine uptake via a 1:1 exchange with glutamate across the cell membrane [13]. Glutamate competitively inhibited cystine uptake into cultured human umbilical vein endothelial cells. Furthermore, glutathione levels decrease as glutamate levels increase [14]. Glutamate cytotoxicity in neuronal cells has been explained relative to the inhibition of cystine transport into the cells by extracellular glutamate [36]. We found here that increased plasma glutamate inhibited cystine uptake through Xc⁻ on endothelial cells, resulting in elevated plasma cystine and decreased glutathione. If so, then decreased glutathione might be responsible for endothelial dysfunction and coronary vasospasm.

Some amino acids other than glutamate and cystine have recently been implicated in endothelial dysfunction. Amino acid metabolism reflecting the activity of arginase, which hydrolyzes L-arginine to ornithine and urea, is increased in patients with type 2 diabetes and is associated with endothelial dysfunction. The ratios of ornithine and proline to citrulline have been associated with the activity of arginase responsible for NO production [18]. Branched-chain leucine, isoleucine and valine reportedly promote endothelial dysfunction through increased ROS generation [19]. However, these amino acids did not significantly differ between the two groups in the present study. Therefore, we consider that only glutamate together with cystine could be a risk factor for VSAP.

The molecular mechanisms responsible for the increase of glutamate levels in patients with VSAP remain unclear. We consider that two mechanisms might be involved in the elevation of glutamate in these patients. The first possible mechanism is glutamate release from vascular cells induced by the vasoconstrictor endothelin-1 (ET-1) derived from endothelial cells. Glutamate levels are significantly higher in pulmonary artery hypertension (PAH) than in control arteries [37]. ET-1 triggers calcium influx in human pulmonary artery smooth muscle cells and glutamate efflux from non-neuronal cells in patients with PAH [38–40]. A selective type A ET-1 receptor antagonist inhibits the effects of ET-1 glutamate release [37]. Levels of ET-1 after acetylcholine or ergonovine provocation tests increase in patients with VSAP [41,42] and an ET-1 gene polymorphism is associated with variant angina in Korean patients [43].

**Table 3.** Multivariate logistic regression analysis of determinants for VSAP.

| Univariate logistic regression | Multivariate logistic regression |
|-------------------------------|--------------------------------|
|                               | Model 1                        | Model 2                        |
|                               | OR 95% CI                      | OR 95% CI                      |
| Age                           | 1.043 0.997–1.092              | 1.036 0.978–1.098              |
| Sex                           | 1.894 0.485–7.400              | 1.172 0.154–8.921              |
| BMI                           | 1.061 0.861–1.308              | 1.325 0.802–2.191              |
| Cr                            | 1.427 0.993–2.049              | 1.463 1.015–2.108              |
| Cystine                       | 2.566 1.214–5.424              | 2.661 1.236–5.730              |
| Glu                           | 1.487 1.053–2.099              | 1.463 1.015–2.108              |

BMI and Cr were respectively analyzed as increases of 1 kg/m² and 0.1 μmol/L, respectively. Age and Glu/Cystine were measured as per year and 10 μmol/L increases, respectively. Male = 1 and female = 2. Model 1 included all clinical and laboratory variables in addition to Glu, but not Cystine. Model 2 included all variables in addition to Cystine, but not Glu.

BMI: body mass index; CI: confidence interval; Cr: creatinine; Glu: glutamate; OR: odds ratio.

**Figure 3.** Association between glutamate and biological antioxidant potential (A) and cystine (B) in patients with VSAP. VSAP: vasospastic angina pectoris.
Therefore, we postulate that glutamate is released from endothelial cells and vascular smooth muscle cells by ET-1 produced by vasospasm or genetic excess ET-1 action. The second pathway might be excessive nutritional intake of glutamate and/or cystine. Monosodium glutamate is a food additive that enhances flavor. Acute ingestion of monosodium glutamate is associated with adverse symptoms such as flushing, sweating, headache and arrhythmia. Plasma glutamate concentrations are temporally elevated after monosodium glutamate ingestion [44]. Moreover, dietary cystine uptake in the intestine and resorption in the kidneys are important for maintaining required levels of this amino acid in humans [13]. The present study found significantly higher creatinine values in the patients with VSAP-positive than VSAP-negative. Reduced renal function might participate in the higher plasma levels of glutamate and cystine in patients with VSAP who do not smoke. Further studies are required to define the mechanisms of elevated glutamate in patients with VSAP who do not smoke.

Limitations

This study has some limitations. We could not show direct evidence of Xc\(^{-}\) action and intracellular glutathione depletion in vascular cells. Such evidence is very difficult to show in vivo; thus, this topic should be investigated in human vascular cells in vitro. The prevalence of VSAP is higher among Japanese than Caucasian patients with coronary heart disease [45]. Paraoxonase 1 and endothelial nitric oxide synthase genes polymorphisms are associated with coronary artery vasospasm in Japanese and Africans [46]. Whether the glutamate and cystine elevations in patients with VSAP who do not smoke are comparable with those in other ethnic groups should be investigated. Finally, the patient cohort was small. Studies of larger populations are required to evaluate whether plasma glutamate and cystine levels are clinically useful risk factors for VSAP.

Conclusion

Plasma glutamate levels were increased in patients with VSAP who showed only epicardial coronary artery spasm and did not smoke, were positively associated with plasma cystine and were negatively associated with the biological antioxidant potential levels. These findings suggest that plasma glutamate might serve as a novel risk factor for VSAP.

Disclosure statement

The authors have no conflict of interest to declare.

Funding

This study was supported by Grants-in-Aid from the Japan Society for the Promotion of Science [grant number: 15K09105 to Y. Kawai].

ORCID

Yasuyuki Kawai http://orcid.org/0000-0002-1901-3755

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