ORIGINAL RESEARCH

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Levels of indoxyl sulfate are associated with severity of coronary atherosclerosis

Abstract

Purpose: Indoxyl sulfate (IS) is linked to endothelial damage, NF- κ B activation and induced development of atherosclerosis. The purpose of this study was to investigate the relationship between serum IS levels and the severity of coronary artery stenosis. In addition, the relationship among IS and various cardiovascular risk factors was also explored.

Methods: Serum IS concentrations were measured using ultra performance liquid chromatography in 191 consecutive patients presenting with stable angina. The associations between serum IS levels and angiographic indexes of the number of diseased vessels, modified Gensini scores and calcium scores were determined.

Results: Patients with significant coronary artery stenosis were found to have higher serum IS levels than patients with normal coronary arteries. Using multivariate analysis, serum IS levels were found to be independently associated with the presence and severity of coronary artery disease (CAD). Furthermore, statistically significant correlation was observed between the serum IS levels and age, Agatston calcium score, volume calcium score, modified Gensini score, coronary lesions, coronary disease and Framingham-10 year risk score.

Conclusion: This study indicates that serum IS levels are significantly higher in the presence of CAD and correlate with the severity of the disease and coronary atherosclerosis scores, which suggest that increased serum IS may be involved in the pathogenesis of coronary atherosclerosis.

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Chronic kidney disease (CKD), which closely correlates with high cardio-vascular disease (CVD) prevalence and mortality, has been a clinical challenge for a long time. There are several common pathogenetic pathways between CKD and coronary artery disease (CAD) and mounting data supports the lethal synergy in CKD patients with CVD [1]. CKD is simply a staging parameter referring to kidney function, whereas the process of vascular damage is the consequence of mediators such as the uremic toxins that are retained as kidney function deterioration; these are the compounds that provide direct toxic effects, causing coronary artery injury [2,3].

Protein-bound uremic retention solutes constitute a group whose common characteristic is their difficult removal by dialysis. Many of these compounds, which accumulate in patients with CKD, have the capacity to interfere with metabolic systems in the body, leading to cardio-vascular damage [4]. Previous studies also show that uremic retention solutes play a role in inflammation and are implicated in the pathogenesis of accelerated atherosclerosis [5,6]. Indoxyl sulphate (IS), a protein-bound uremic retention solute, is one of the organic anions metabolized in the liver from indole, which is produced by intestinal bacteria as a metabolite of tryptophan [7]. Dou et al. demonstrated that IS and p-cresol suppress both wound repair and the proliferation of endothelial cells (ECs) that are derived from the human umbilical vein [8]. IS also enhances the production of reactive oxygen species (ROS), increases NAD(P)H oxidase activity and decreases glutathione levels in these cells [9]. Yamamoto et al. [10] showed that IS causes vascular smooth muscle cells (VSMCs) to proliferate via activation of extracellular-regulated protein kinase (ERK). Motojima et al. [11] showed that IS induces plasminogen activator inhibitor-1 (PAI-1) and activates nuclear factor-kappaB (NF- κ B) in human renal proximal tubular cells. In addition, our previous study demonstrated that serum IS levels were elevated in patients with type 2 diabetes and CAD. This increase was associated with renal function deterioration, inflammation and coronary atherosclerosis [12].

Because the pathogenesis of atherosclerosis involves the dysfunction of vascular ECs, proliferation of VSMCs [13-15] and activation of NF- κ B [11], which, in turn, up-regulates PAI-1 expression [16], these findings indicate that IS induces the initiation and/or progression of atherosclerosis. Although such evidence of the biological toxicity of IS support its possible role as a cardiovascular risk factor, there is paucity of data on the association between IS levels and the severity or extent of CAD. In the present study, which is aimed at investigating this hypothesis, serum IS levels and angiographic indexes of the number of diseased vessels, calcium scores, and modified Gens-

ini score (as measured by cardiac multi-slice computed tomography) were assessed in a cohort of stable angina patients. The possible association between IS levels and selected cardiovascular risk factors were also examined in this patient population.

Methods

Study population

The study population consisted of 191 consecutive patients who underwent first-time angiography with clinical diagnosis as suspected stable angina from June 2007 to June 2009 at the Cardiovascular Clinic of E-Da Hospital. Stable angina pectoris was defined as effort-related chest pain without evidence of recent deterioration or rest pain within the previous six months. The patients with histories of concomitant inflammatory diseases (such as infection, sepsis, malignancy, liver disease and collagen disease), steroid use or surgery within the month prior to admission, on the basis of the interview, physical examination, biochemistry lab data and urinalysis, were excluded from the study. The study protocol was approved by the Human Research Ethics Committee of the hospital, and informed consent was obtained from each patient.

Laboratory measurements

In all cases, blood was drawn for the measurement of IS, centrifuged and the supernatant was transferred to a separate container before storing. Before coronary angiography, serum creatinine, uric acid and lipid profiles were measured via standard commercial methods as described previously [17]. Renal function was defined on the basis of previously estimated glomerular filtration rate (eGFR) values, which were calculated according to the extended Modification of Diet in Renal Disease (MDRD) formula [18]. Prior to determination of IS, serum samples were deproteinized by adding three parts methanol to one part serum. All analyses were performed using a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Milford, MA, USA) as described previously [12,19].

Angiographic Definitions

Coronary angiograms were obtained according to standard techniques and the severity of stenosis was assessed using quantitative coronary angiography. Angiograms were obtained and quantitative coronary angiographic analysis was performed by at least two experienced interventional cardiologists blinded to clinical information and serologic parameters. The possible scores for severity of stenosis ranged from 0 to 3 diseased ves-

sels. The possible scores of this index ranged from 0 to 3 diseased vessels. The criterion for 1-, 2-, or 3-vessel disease was $\geq 50\%$ luminal reduction of the right coronary artery. The stenosis of the left main coronary artery did not exceed 50%. The modified Gensini index is the sum of the total weights for each segment [20].

Agatston scores were determined using CAC-scoring software (SmartScore, GE Healthcare, San Francisco, CA, USA). The Agatston calcium score was obtained by multiplying the area of coronary calcification by a weighting factor dependent on the peak signal anywhere in the lesion. The scores for individual lesions were added to obtain the Agatston calcium score for the entire coronary tree. The total calcium volume was calculated by multiplying the area of the calcified lesion (measured in square millimeters) by section thickness.

Statistical analysis

Data normality was analyzed using the Kolmogorov-Smirnov test. Continuous, normally distributed variables were presented as mean $\pm$ SD and non-normally distributed variables as median (inter-quartile range). Statistical differences in variables were compared using unpaired Student's *t*-tests for normally distributed variables. Categorical variables were recorded as frequencies and/or percentages, and inter-group comparisons were analyzed by chi-square test. Since the distribution of serum IS, and triglycerides were skewed, logarithmically transformed values were used for statistical analysis, with significant differences being determined using unpaired Student's *t*-tests. Using multiple logistic regression, these variables were assessed for independent associations with the presence of CAD. Association of IS and severity of CAD were analyzed by logistic regression, using the non-diseased vessel group as a reference. For stratified analysis, the multivariable-adjusted odds ratio (OR) associated with a doubling in severity of coronary artery disease was calculated. Multivariate adjusted ORs are presented with 95% confidence interval (CI). The Spearman correlation analysis was used to examine correlations between serum IS and the values of cardiovascular risk factors. Statistical significance was accepted if $p < 0.05$. All of the statistical analyses were performed using the SAS statistical software, version 8.2 (SAS Institute; Cary, NC, USA).

Results

Patients' baseline clinical, biochemical and angiographic characteristics are listed in Table 1. There was no statistically significant difference in serum IS levels between men and women. Patient groups with and without traditional cardiovascular risk

factors did not have significantly different serum IS levels, with one exception: patient groups with and without diabetes showed a significant difference in serum IS levels (data not shown). A statistically significant association was observed between age and serum IS concentration ($\beta = 0.327$, $p < 0.0001$, $n = 191$).

Seventy-four patients were categorized as having no CAD, while 117 patients were categorized as having 1-, 2-, or 3-vessel disease (Table 1). Median serum total IS levels in patients with CAD were significantly higher than levels in patients without CAD (0.55 mg/L [interquartile range 0.23 to 1.53] vs. 0.50 mg/L [interquartile range 0.23 to 0.83], $p = 0.026$, Table 1). A statistically significant difference in total IS levels was seen among patients categorized according to the number of diseased vessels ($p < 0.05$). As expected, CAD subjects were predominantly male, older, smokers, with a higher incidence of hypertension, hyperlipidemia and diabetes mellitus, higher levels of fasting sugar, triglyceride, uric acid and creatinine and higher Gensini scores than those of non-CAD control subjects. CAD subjects also had lower total cholesterol, HDL-C, albumin levels and estimated GFR than those of non-CAD control subjects, while BMI, systolic blood pressure, diastolic blood pressure, triglyceride, LDL-C and total white blood cell count were not significantly different between the two groups.

Multivariate logistic regression analysis, including traditional cardio-vascular risk factors and biochemical measurements, revealed that body mass index, hypertension, diabetes, HDL-C, uric acid, eGFR and IS were all independently associated with CAD (Table 2). Moreover, simple and multivariable logistic regression analysis found that serum IS levels were associated with the number of diseased vessels. Multiple logistic regression analysis in fully adjusted odds ratios for number of diseased vessels in the one-vessel, two-vessel and three-vessel patients were 0.351 (95% CI, 0.059 – 2.093), 3.011 (95% CI, 1.004 – 9.751) and 3.076 (95% CI, 1.138 – 8.310), respectively (Table 3).

Spearman correlation analysis revealed that IS was positively associated with age, Agatston calcium score, volume calcium score, modified Gensini score, coronary lesions, coronary disease and Framingham-10 year risk score. In addition, eGFR was negatively correlated with levels of IS (Table 4 and Figure 1A and 1B).

Discussion

The present study demonstrated for the first time that serum IS levels were positively and significantly correlated with the plaque burden of modified Gensini score in patients who underwent first-time coronary angiography for suspected CAD.

TABLE 1. Baseline clinical and biochemical characteristics of the study population

Variable	Total	CAD Present	CAD Absent	p value
No	191	117	74	
Sex, male/female	111/80	86/31	25/49	<.0001
Age (years)	62.6±10.2	64.8±10.2	59.2±9.4	0.0002
Body mass index (kg/m ²)	25.4±4.0	25.8±4.0	24.8±3.9	0.099
Hypertension (n, %)	135(70.7)	101(86.3)	34(46.0%)	<.0001
Hyperlipidemia (n, %)	120(62.8)	86(73.5)	34(46.0)	0.0001
Diabetes mellitus (n, %)	60(31.4)	53(45.3)	7(9.5)	<.0001
Current smoking (n, %)	67(35.3)	55(47.0)	12(16.4)	<.0001
Systolic blood pressure (mmHg)	134±22	135±22	132±22	0.457
Diastolic blood pressure (mmHg)	76±11	75±10	77±12	0.157
Fasting glucose (mg/dl)	123.0±54.0	138.4±62.7	100.1±23.5	<.0001
T-cholesterol (mg/dl)	181.2±47.7	171.5±42.2	196.3±51.9	0.0004
Triglyceride (mg/dl)*	107.0(75.0-155.3)	116.5(85.3-172.8)	94.5(64.3-136.3)	0.014
HDL-cholesterol (mg/dl)	42.0±13.2	38.5±11.6	48.0±13.8	<.0001
LDL-cholesterol (mg/dl)	110.3±39.2	106.2±37.5	117.3±41.4	0.065
Uric acid (mg/dl)	6.3±1.8	6.8±1.9	5.7±1.4	<.0001
Creatinine (mg/dl)	1.20±0.49	1.37±0.53	0.93±0.25	<.0001
Albumin (g/dl)	4.0±0.4	4.0±0.4	4.1±0.3	0.040
eGFR (ml/min/1.73m ²)	65.9±20.3	57.6±16.9	79.1±18.2	<.0001
White blood cell count (×10 ⁹ /l)	6.657±2.017	6.849±2.135	6.361±1.794	0.108
Indoxyl sulfate (mg/l)*	0.49(0.23-1.25)	0.55(0.23-1.53)	0.50(0.23-0.83)	0.026
No. of diseased vessels				
0		0	74	
1		29		
2		37		
3		51		
Location of stenosis				
Left anterior descending coronary artery		98/191		
Left circumflex coronary artery		80/191		
Right coronary artery		78/191		
Gensini score	15.5(0.0-56.0)	36.0(17.0-83.0)	0.0(0.0-0.0)	<.0001

Data are expressed as mean ± SD, number (percentage), or median (interquartile range). *: Significant difference was tested using log-transformed data. CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein. eGFR, estimated glomerular filtration rate.

TABLE 2. Association of risk of coronary artery disease with indoxyl sulfate and other cardiovascular risk factors

	Odds ratio	95% Confidence Interval	p value*
Body mass index	1.12	1.03-1.23	0.012
Hypertension	8.79	3.82-20.23	<.0001
Diabetes	7.71	2.98-19.89	<.0001
Total cholesterol	0.99	0.98-1.00	0.055
Triglyceride	1.00	0.99-1.00	0.644
HDL-cholesterol	0.95	0.92-0.98	0.001
LDL-cholesterol	0.99	0.99-1.00	0.174
Albumin	0.58	0.21-1.60	0.290
Uric acid	1.40	1.12-1.75	0.003
Indoxyl sulfate	1.41	1.02-2.31	0.041
eGFR	0.92	0.89-0.95	<.0001
Smoking	2.36	0.92-6.03	0.074

*: Adjusted for age and sex by multiple logistic regression analysis. HDL, high-density lipoprotein; LDL, low-density lipoprotein. eGFR, estimated glomerular filtration rate.

TABLE 3. The impact of serum indoxyl sulfate levels on the severity of coronary artery disease, by univariate and multivariate analysis

Factor	Severity of coronary artery disease			
	Non-coronary artery disease	Single vessel disease	Two vessel disease	Three-vessel disease
Indoxyl sulfate				
Crude OR	1.00 (reference)	0.910	2.498	2.927
Adjusted OR*	1.00 (reference)	0.351	3.011	3.076
95% CI	-	0.059-2.093	1.004-9.751	1.138-8.310
p value	-	0.351	0.047	0.027

*: The confounders adjusted for in this model are sex, age, body mass index, fasting glucose, systolic blood pressure, diastolic blood pressure, total cholesterol, high-density lipoprotein, low-density lipoprotein, triglyceride, and smoking status. OR, Odds ratio; CI, Confidence interval.

A significant and positive correlation was also observed between serum IS concentrations and Agatston calcium score, volume calcium score, coronary lesions and Framingham-10 year risk score. Another finding of our study also showed that patients with angiographically-confirmed CAD had significantly higher levels of IS compared with patients without CAD. Moreover, serum IS levels were independently associated with the presence and severity of CAD and serum IS was directly and negatively associated with eGFR. Although the biological mechanisms involving IS in the pathogenesis of CAD

are not well understood, our previous study suggests that IS plays a crucial role in the inflammatory process and in the development of coronary atherosclerosis [12]. Patients with higher IS levels may, therefore, have greater inflammatory tendency and be more vulnerable to CAD development [12].

IS was initially believed to be a uremic retention solute resulting from renal function deterioration. The overloading of IS in CKD rats results in glomerular sclerosis and interstitial fibrosis [7] via aberrant genetic expression of TGF- β 1, TIMP-1 and Pro- α 1 collagen [21,22], as well as complex redox altera-

TABLE 4. Spearman rank correlation coefficients between indoxyl sulfate and cardiovascular risk factors, calcium score, Gensini score, coronary disease and Framingham risk score

Variable	r	p value
Age	0.455	<.0001
Total cholesterol	-0.034	0.657
Triglyceride	-0.001	0.995
HDL-cholesterol	-0.143	0.065
LDL-cholesterol	-0.042	0.590
eGFR	-0.382	<.0001
Agatston calcium score	0.315	0.011
Volume calcium score	0.287	0.022
Gensini score	0.155	0.033
Coronary lesions (>50% diameter stenosis)	0.205	0.007
Coronary disease	0.197	0.020
Framingham-10 year risk score	0.382	<.0001

HDL, high-density lipoprotein; LDL, low-density lipoprotein; eGFR, estimated glomerular filtration rate.

tions [23]. IS also causes endothelial and vascular dysfunction by promoting vascular smooth muscle cell proliferation [15] through activation of platelet-derived growth factor receptors [15] and mitogen-activated protein kinase pathways [10]. Clinically, IS is associated with increased aortic calcification and vascular stiffness [2]. Another study also demonstrates a gradual, but significant, increase in serum IS as the CKD stage worsens and this has been shown to be associated with cardiovascular mortality risk [2]. Interestingly, results on the association between serum IS and coronary atherosclerotic severity, calcium scores and Framingham-10 year risk score are consistent with previous reports of IS association with endothelial damage and cardiovascular risk [2,9].

spite a significant association between high-serum IS and coronary atherosclerosis progression, the mechanisms of how IS promotes disease remain to be elucidated. It has been demonstrated that IS as a pro-inflammatory factor by inducing endothelial dysfunction [8,9,24]. Faure et al. have reported that IS induces endothelial damage by increasing the production of human umbilical vein endothelial cell (HUVEC) micro-particles and inhibiting HUVEC proliferation and migration [24]. IS synergistically enhances ROS production and PDGF-induced proliferation and migration of VSMCs, which indi-

FIGURE 1. Association between serum indoxyl sulfate concentrations and Gensini score and Framingham-10 year risk score. Serum indoxyl sulfate concentrations had a significant and positive association with Gensini scores (A) and Framingham-10 year risk score (B).

cates that IS stimulates the pathogenesis of atherosclerosis [15]. Regardless of whether or not IS induces free radicals or micro-particle production, inhibits endothelial cell proliferation, activates NADPH oxidase, which decreases the antioxidant defense and enhances smooth muscle cell proliferation, these are all major contributors to the pathogenesis of endothelial dysfunction, which is the initial step towards coronary atherosclerosis.

There are some limitations of this study that need to be considered. The cross-sectional design limits the ability to infer a causal relationship between increased serum IS levels and CAD. A single measurement of blood IS does not enable analysis of the relationship over time. Long-term follow-up, to clarify the role of serum IS in association with CAD, is warranted. Moreover, in the *in vivo* study, whether IS induces CAD is related to endothelial dysfunction (i.e., adhesion molecule enhancement, inflammatory effects of vessel and oxidative over-stress or coagulant activity increase) remains unclear. Further investigation is needed.

In conclusion, elevated IS levels in stable angina subjects are associated with an increase in the number of diseased coronary vessels and plaque burden, as measured by Gensini score and Framingham-10 year risk score. This association indicates that IS is not only a good candidate for a uremic-specific pathogen but may also play a role in the development and severity of coronary atherosclerosis and future cardio-vascular risk prediction.

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