Derivatives of Reactive Oxygen Metabolites Correlates with High-Sensitivity C-Reactive Protein

Fumihiko Kamezaki, Kazuhito Yamashita, Takahiro Kubara, Yoshiyuki Suzuki, Seiya Tanaka, Ryouji Kouzuma, Masahiro Okazaki, Hiromi Tasaki, and Yutaka Otuji

The Second Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan.

Aim: High-sensitivity C-reactive protein (hsCRP) is a predictor of cardiovascular events. Although oxidative stress may also be related to cardiovascular disease, there are few studies comparing the two. We therefore examined the association of hsCRP, serum lipids, and derivatives of reactive oxygen metabolites (D-ROMs) in coronary artery disease.

Methods: We measured the levels of serum lipids, hsCRP, plasma brain natriuretic peptides (BNP) and D-ROMs in 131 consecutive patients undergoing cardiac catheterization. We divided these subjects into three groups according to their levels of hsCRP.

Results: In group C (hsCRP > 3.0 mg/L), mean levels of serum D-ROMs were significantly higher than in groups A (hsCRP < 1.0 mg/L) and B (hsCRP 1.0 to 3.0 mg/L). Serum levels of D-ROMs and log (hsCRP) correlated in the total population (r = 0.479, p < 0.0001), and D-ROMs, HDL-C, LDL-C and log-transformed plasma BNP were independent predictors of hsCRP (p < 0.0001).

Conclusion: We concluded that oxidative stress increases in patients at high risk for cardiovascular events based on their hsCRP.


Key words; D-ROMs, hsCRP, Oxidative stress, Inflammation

Introduction

Inflammation is important in atherosclerosis and cardiovascular disease. High-sensitivity C-reactive protein (hsCRP) is an inflammatory marker that can indicate the presence of acute coronary syndrome and stable coronary artery disease (CAD). The AHA/CDC group divided hsCRP levels into 3 groups: concentrations of < 1.0 mg/L were low risk, 1.0 to 3.0 mg/L were average risk, and > 3.0 mg/L were high risk. Patients in the high-risk group had a two-fold greater relative risk of developing cardiovascular disease than those in the low-risk group.

Oxidative stress may also be a risk factor for CAD and a predictor of cardiovascular mortality. Biomarkers of oxidation, such as serum malondialdehyde (MDA) levels measured as thiobarbituric acid reactive substances (TBARS), oxidized low-density lipoprotein (oxLDL), and urinary excretion of 8-iso-prostaglandin (PG) F2 alpha, are strongly predictive of cardiovascular events in patients with stable CAD; however, these assays are not usable in clinical settings because the methodology is complex and the analytes are unstable.

Here, we used a simpler method of detecting hydroperoxide (ROOH) levels by measuring derivatives of reactive oxygen metabolites (D-ROMs). Hydroperoxides are converted into radicals that oxidize N,N-diethyl-para-phenylenediamine and can be detected spectrophotometrically. We therefore evaluated the association of D-ROMs with hsCRP and coronary atherosclerosis in patients undergoing coronary angiography.
Methods

Study Subjects
Among 164 consecutive patients undergoing cardiac catheterization, 131 were eligible for this study. Patients with acute coronary syndrome, valvular heart disease, idiopathic dilated or hypertrophic cardiomyopathy, severe chronic renal failure (serum creatinine > 2.0 mg/dL), chronic inflammatory disease, and malignant disease were excluded from the study. Five patients with levels of hsCRP in serum > 10 mg/L were also excluded. All 131 patients underwent coronary angiography because they were suspected of having CAD. On the basis of hsCRP levels, we divided these patients into three groups: group A, serum level < 1.0 mg/L; group B, 1.0-3.0 mg/L; and group C, > 3.0 mg/L.

The institutional ethics committee approved this study, and written informed consent was obtained from each patient before the study.

Laboratory Measurements
After admission, venous blood samples were collected after an overnight fast and no cigarette smoking. Measurements such as plasma lipids, serum hsCRP, and creatinine, except D-ROMs, were performed immediately using automatic measurement devices in the central measurement laboratory of our hospital. The lower limit of hsCRP detection was 0.2 mg/L. The concentrations of plasma brain natriuretic peptides (BNP) were determined by radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) (SRL Inc., Tokyo, Japan). The sensitivity of the assay for BNP was 2.0 pg/mL. For D-ROMs, serum was isolated at 4°C and, after centrifugation, kept frozen at -80°C until analysis. The estimated glomerular filtration rate (eGFR) was calculated by applying the Cockcroft-Gault formula to creatinine concentration, with adjustments made for female patients. Urinary 8-isoprostane levels were determined by radioimmunoassay (EIA) (BML Inc., Tokyo, Japan). The sensitivity of the 8-isoprostane assay was 12.3 pg/mL. All urinary measurements were corrected for recovery and creatinine excretion.

Derivatives of Reactive Oxygen Metabolites (D-ROMs)
A commercially available method was used to assess the levels of oxidative stress in serum (d-ROMs test; Diacron, Grosseto, Italy). The test is based on the concept that the amount of organic hydroperoxides present in serum is related to the free radicals from which they are formed. When the serum sample is dissolved in acidic buffer, the hydroperoxides react with the transition metal ions liberated from the proteins in the acidic medium and are converted to alkoxide and peroxy radicals. These newly formed radicals are able to oxidize an additive (N,N-diethyl-para-phenylen-diamine) to the corresponding radical cation. The concentration of this persistent species can easily be determined spectrophotometrically (absorption at 505 nm). The normal values of the test are between 250 and 300 U.CARR. (Carratelli Units), where 1 U.CARR. corresponds to 0.8 mg/L H2O2. All samples from one treatment sequence were analyzed together. The variability of within-day and between-day assays was 0.3-6.6% (n=8) and 0.3-5.1% (n=8), respectively.

Coronary Angiography and Echocardiography
Cardiac catheterization was performed with all patients in the fasted state after receiving 5 mg of diazepam orally. Coronary angiography (CAG) was performed using a femoral, brachial, or radial approach with 5- or 6-French Judkins left and right 4.0 catheters; two angiographers judged the degree of stenosis. When there was no agreement between the two, a third angiographer was consulted for the final decision. Stenosis of 75% or greater, as described by the American Heart Association (AHA) classification, was considered significant. The degree of stenosis was expressed by a coronary score using the Gensini method. Echocardiographic evaluation was also performed before coronary angiography in all patients.

Statistical Analysis
Continuous data are expressed as the means ± standard deviations (SDs) or median (25%, 75% interquartile). HsCRP and BNP were log-transformed to achieve linearity. Categorical data are presented as absolute values and percentages. Comparison of continuous variables among the three groups was performed by one-way ANOVA, with post-hoc comparisons with Tukey-Kramer multiple tests. The Chi-squared and Fisher’s exact tests were used to compare categorical variables as appropriate. The linearity of the relationship between two variables was assessed by linear regression. Multivariate analysis was performed using stepwise linear regression. Variables that remained significant at \( p < 0.25 \) were retained for the final model. A \( p \) value < 0.05 was considered significant. Analyses were conducted using JMP IN statistical software (Version 5.1.2, SAS Institute Inc., Cary, North Carolina).
Patient Characteristics and Serum D-ROMs Levels

The mean age of patients was 69 ± 9 years old, and 69% were male (Table 1). The mean level of D-ROMs in serum was 333 ± 63 U.CARR., and the median value of hsCRP was 1.40 (25th and 75th inter-quartile; 0.60 and 3.00) mg/L. Patient groups divided by hsCRP showed significant differences in plasma BNP, plasma HDL-cholesterol, and eGFR (p < 0.0001, p = 0.0208 and p = 0.0359, respectively). There were no differences in patient medications by group, but patients in group C more frequently had a history of congestive heart failure (p < 0.05).

Mean values of serum D-ROMs were 299 ± 46 U.CARR. in group A, 341 ± 60 U.CARR. in group B, and 373 ± 66 U.CARR. in group C (p < 0.0001 by one-way ANOVA) and varied within each group (Fig. 1), but urinary 8-isoprostane levels, another oxidative stress marker, did not change (Table 1).

Hemodynamic Data and Coronary Angiography

There were no differences in blood pressure, heart rate, numbers of significantly stenosed coronary arteries, or Gensini scores among the three groups (Table 2); however, ejection fraction measured by echocardiography in group B was lower than in group A (p < 0.05).

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Total Group (n = 131)</th>
<th>Group A (hsCRP &lt; 1; n = 52)</th>
<th>Group B (hsCRP = 3; n = 47)</th>
<th>Group C (hsCRP &gt; 3; n = 32)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>90 (69)</td>
<td>38 (73)</td>
<td>32 (68)</td>
<td>20 (63)</td>
<td>0.5950</td>
</tr>
<tr>
<td>Age (years)</td>
<td>69 ± 9</td>
<td>69 ± 8</td>
<td>69 ± 11</td>
<td>72 ± 10</td>
<td>0.2941</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>23.3 ± 5.1</td>
<td>24.0 ± 2.7</td>
<td>23.4 ± 3.5</td>
<td>22.9 ± 2.8</td>
<td>0.2449</td>
</tr>
<tr>
<td>Hs-CRP (mg/L)</td>
<td>1.40 (0.60, 3.00)</td>
<td>0.55 (0.33, 0.70)</td>
<td>1.60 (1.20, 2.20)</td>
<td>6.5 (4.03, 11.85)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>BNP (pg/mL)</td>
<td>59 (18, 177)</td>
<td>29 (15, 89)</td>
<td>66 (16, 203)</td>
<td>102 (56, 479)</td>
<td>0.0009</td>
</tr>
<tr>
<td>8-isoprostane (pg/mg·cr)</td>
<td>166 ± 84</td>
<td>153 ± 73</td>
<td>174 ± 97</td>
<td>174 ± 81</td>
<td>0.3653</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD or number (%). ¶ hsCRP and BNP are expressed as the median (25%, 75% interquartile).

* p < 0.05 vs Group C (one-way ANOVA followed by the Tukey-Kramer multiple comparison test)

Hs-CRP, high-sensitivity C reactive protein; BNP, brain natriuretic peptides; eGFR, estimated glomerular filtration rate; MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary aorta bypass graft; CHF, congestive heart failure; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker
Correlation between hsCRP and other Variables

Serum D-ROMs showed a significant positive correlation to log-transformed serum hsCRP ($r = 0.479$, $p < 0.0001$) (Fig. 2), log-transformed plasma BNP, and TC/HDL-C, and showed a negative correlation to HDL-C and the ejection fraction (Table 3). In multivariate linear regression analysis of hsCRP (adjusted $R^2 = 0.290$), D-ROMs, HDL-C, LDL-C and log-transformed plasma BNP were independent predictors of hsCRP ($p < 0.0001$). Serum levels of D-ROMs were the most strongly predictive of serum hsCRP ($\beta = 0.364$, $F = 20.6$, $p < 0.0001$).

Discussion

In our study, we found that (1) serum levels of D-ROMs, a marker of oxidative stress, were significantly higher in patients in the hsCRP-defined high-risk group for cardiovascular events; (2) levels of serum hsCRP and D-ROMs positively correlated; (3) D-ROMs was an independent predictor of hsCRP levels. These findings suggest that both inflammation and oxidative stress increase in patients at high risk for cardiovascular events. Additionally, measuring D-ROMs in vivo with a simple, inexpensive test makes it possible to identify subjects with a high level of oxidative stress who are at high risk for cardiovascular events.

Oxidative Stress and D-ROMs

Other oxidative markers, including oxidized LDL in serum, TBARS in plasma, and urinary 8-isoprostanate may correlate with coronary atherosclerosis and cardiovascular events, but TBARS and 8-isoprostanate measurements are complicated and oxLDL is unstable, rendering these tests impractical for clinical use.

Here, we measured derivatives of reactive oxygen metabolites in serum with the D-ROM test. In this method, hydroperoxides are converted into radicals that oxidize N,N-diethyl-para-phenylenediamine, which can be detected spectrophotometrically, even in the clinic. Serum levels of D-ROMs are between 200 to 300 U.CARR. in normal volunteers and are greater in subjects who smoke, are on hemodialysis, or who are hypertensive.

We found elevated D-ROMs in patients with CAD and coronary risk factors. Interestingly, elevated levels of plasma 8-isoprostanate, another marker of oxidative stress, are associated with the extent and severity of CAD. In another study of 206 subjects, levels of TBARS correlated with disease severity as determined by angiography; however, we did not

Table 2. Hemodynamic data and coronary angiography

<table>
<thead>
<tr>
<th></th>
<th>Group A (hsCRP &lt; 1; n = 52)</th>
<th>Group B (1 ≤ hsCRP &lt; 3; n = 47)</th>
<th>Group C (hsCRP ≥ 3; n = 32)</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>133 ± 21</td>
<td>136 ± 24</td>
<td>137 ± 28</td>
<td>0.6787</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>69 ± 13</td>
<td>73 ± 12</td>
<td>70 ± 16</td>
<td>0.3276</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>69 ± 12</td>
<td>72 ± 11</td>
<td>70 ± 12</td>
<td>0.3030</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>68 ± 11</td>
<td>60 ± 16*</td>
<td>63 ± 12</td>
<td>0.0247</td>
</tr>
<tr>
<td>Coronary angiography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/1/2/3 vessel disease (n)</td>
<td>14/15/15/8</td>
<td>8/12/16/11</td>
<td>10/5/10/7</td>
<td>0.5991</td>
</tr>
<tr>
<td>Multivessels (n (%))</td>
<td>23 (44)</td>
<td>27 (57)</td>
<td>17 (41)</td>
<td>0.4072</td>
</tr>
<tr>
<td>Gensini Score</td>
<td>36.5 (15.6, 60.8)</td>
<td>37.0 (17.5, 49.0)</td>
<td>34.8 (8.9, 90.6)</td>
<td>0.9232</td>
</tr>
</tbody>
</table>

Data are the mean ± SDs or numbers (%). Gensini score is expressed as the median (25%, 75% interquartile). Ejection fraction was measured by echocardiography. *$p < 0.05$ vs Group A. Hs-CRP, high-sensitivity C reactive protein.
find a correlation between D-ROM levels and CAD severity, which may indicate an indirect relationship between this marker and the expression of oxidative damage in patients with stable CAD. We also found no association between serum hsCRP and the severity of CAD, as reported previously. HsCRP is associated with plaque instability and systemic inflammation\(^\text{20, 21}\) and D-ROMs may also reflect a systemic chronic oxidative state.

**CRP and Oxidative Stress**

Reactive oxygen species (ROS) contribute to vascular disease, including atherosclerosis\(^\text{22}\). These ROS initiate processes involved in atherogenesis through several important enzyme systems, including xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and nitric oxide synthase\(^\text{23}\). Moreover, CRP levels predict the risk for acute coronary syndrome\(^\text{24}\) and for future myocardial infarction and stroke in apparently healthy men\(^\text{9}\). CRP is expressed in atherosclerotic lesions\(^\text{25, 26}\) and may contribute to the instability of atherosclerotic plaques. CRP binds to oxidized LDL and oxidized phosphatidylcholine, but does not bind to native, non-oxidized LDL nor to non-oxidized phosphatidylcholine\(^\text{27}\). CRP frequently colocalizes with p22phox, an essential component of NADH/NADPH oxidase, and CRP increases the expression of p22phox protein and intracellular ROS generation in smooth muscle cells\(^\text{28}\); thus, CRP may be an inflammatory marker and stimulate ROS generation. The strong association between hsCRP and D-ROMs in our study supports this hypothesis. Thus, CRP may increase the production of oxygen radicals via NADH/NDPH oxidase in situations when oxidative stress is already high, leading to a feed forward loop between CRP and oxidative stress.

**Fig. 2.** Relationship between serum D-ROMs levels and log-transformed high-sensitivity CRP.

A significant positive correlation was found between serum D-ROMs levels and log-transformed high-sensitive CRP (\(r=0.479\) and \(p<0.0001\)).

**Table 3.** Univariate and Multivariate Analysis of hsCRP

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate Correlation Coefficient</th>
<th>(p) value</th>
<th>(\beta)</th>
<th>(F) value</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.098</td>
<td>0.2678</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Mass Index (kg/m(^2))</td>
<td>-0.084</td>
<td>0.3397</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>-0.094</td>
<td>0.2840</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>-0.021</td>
<td>0.8108</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>0.142</td>
<td>0.1074</td>
<td>0.163</td>
<td>4.3</td>
<td>0.0399</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>-0.230</td>
<td>0.0082</td>
<td>-0.169</td>
<td>5.0</td>
<td>0.0267</td>
</tr>
<tr>
<td>TC/HDL-C</td>
<td>0.228</td>
<td>0.0087</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBS (mg/dL)</td>
<td>0.043</td>
<td>0.6211</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.036</td>
<td>0.6815</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR (mL/min)</td>
<td>-0.119</td>
<td>0.1770</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-ROMs (U.CARR.)</td>
<td>0.479</td>
<td>&lt;0.0001</td>
<td>0.364</td>
<td>20.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Brain Natriuretic Peptides (pg/mL)</td>
<td>0.300</td>
<td>0.0005</td>
<td>0.240</td>
<td>8.7</td>
<td>0.0038</td>
</tr>
<tr>
<td>8-isoprostane (pg/mg·ct)</td>
<td>0.062</td>
<td>0.4868</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>-0.179</td>
<td>0.0492</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HsCRP and BNP were log-transformed.

Hs-CRP: high-sensitivity C reactive protein; TC: total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FBS, fasting blood sugar; HbA1c, glycohemoglobin; eGFR, estimated glomerular filtration rate; D-ROMs, derivatives of reactive oxygen metabolites; LVEF, left ventricular ejection fraction; BNP, brain natriuretic peptides
Clinical Implications

hsCRP can also indicate unstable angina and acute myocardial infarction\textsuperscript{2,30}, but with a lower odds ratio than other risk factors\textsuperscript{31}; however, the combination of hsCRP and the TC/HDL-C ratio carries a greater projected risk\textsuperscript{31}, and cardiovascular event-free survival rates were lower in the high CRP and high LDL groups than in others\textsuperscript{32}. Thus, the entire adult population does not require screening for hsCRP to assess cardiovascular risk\textsuperscript{33}, but in the future it may be part of a battery of tests for predicting risk\textsuperscript{33}. In particular, the D-ROM test provides a simple, inexpensive, and practical method of identifying subjects with high levels of oxidative stress. Further investigations are needed to determine whether hsCRP combined with D-ROMs is a reliable predictor of cardiac risk.

Study Limitations

The present study has some important limitations. First is the small sample size. Second, D-ROMs cannot elucidate the source of hydroperoxides in serum; however, since hydroperoxides are stable metabolites produced by ROS, D-ROMs should reflect the state of systemic oxidative stress. Third, we only compared serum D-ROMs levels with one oxidative marker, the urinary 8-isoprostane level, and they did not correlate well. This difference could result from differences in samples (blood versus urine) or variability created by moderate alcohol consumption, which can lower hsCRP but increases systemic oxidative stress\textsuperscript{33,34}. Fourth, we did not compare the association between D-ROMs and the clinical endpoint of cardiovascular disease (CVD).

Conclusion

Oxidative stress was significantly greater in patients in the group at high risk for cardiovascular events according to their serum hsCRP levels. Measuring derivatives of reactive oxygen metabolites (D-ROMs) \textit{in vivo} with a simple, inexpensive test makes it possible to identify subjects who are at high risk for cardiovascular events in relation to high levels of oxidative stress.

References

3) Ridker PM: High-sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary preven-
16) Incandela L, Belcaro G, Cesarone MR, De Sanctis MT, Griffin M, Caccio M, Nicolaides AM, Buccioni M, Barsotti A, Martines G, Cornelli U, and Di Renzo A: Oxygen-free radical decrease in hypertensive patients treated with le-
canidipine. Int Angiol, 2001; 20:136-140
18) Vassalle C, Botto N, Andreassi MG, Berti S, and Biagini A: Evidence for enhanced 8-isoprostane plasma levels, as index of oxidative stress in vivo, in patients with coronary artery disease. Coron Artery Dis, 2003; 14:213-218
25) Venugopal SK, Devaraj S, and Jialal I: Macrophage conditioned medium induces the expression of C-reactive protein in human aortic endothelial cells: potential for paracrine/autocrine effects. Am J Pathol, 2005; 166:1265-1271