Original Article

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

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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.

J Atheroscler Thromb, 2008; 15:206-212.

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

Introduction

Inflammation is important in atherosclerosis and cardiovascular disease¹⁾. 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)²⁻⁴⁾. The AHA/CDC group⁵⁾ 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 group⁵⁾.

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)⁶⁾, oxidized low-density lipoprotein (oxLDL)⁷⁾, and urinary excretion of 8-iso-prostaglandin (PG) F2 alpha⁸⁾, 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)⁹⁾. Hydroperoxides are converted into radicals that oxidize N,N-diethyl-para-phenyldiamine and can be detected spectrophotometrically. We therefore evaluated the association of D-ROMs with hsCRP and coronary atherosclerosis in patients undergoing coronary angiography.

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Received: September 14, 2007

Accepted for publication: March 17, 2008

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 excluded⁵⁾. All 131 patients underwent coronary angiography because they were suspected of having CAD. On the basis of hsCRP levels⁵⁾, 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 patients¹⁰⁾. 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)⁹⁾. 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 alkoxy and peroxy radicals. These newly formed radicals are able to oxidize an additive (N,N-diethyl-*para*-phenylenediamine) 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 H_2O_2 . 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 method¹¹⁾. Echocardiographic evaluation was also performed before coronary angiography in all patients.

Statistical Analysis

Continuous data are expressed as the means $\pm$ 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).

Table 1. Patient characteristics

	Total Group (<i>n</i> = 131)	Group A (hsCRP < 1; <i>n</i> = 52)	Group B (1 = hsCRP = 3; <i>n</i> = 47)	Group C (hsCRP > 3; <i>n</i> = 32)	<i>p</i> value
Male (%)	90 (69)	38 (73)	32 (68)	20 (63)	0.5950
Age (years)	69 ± 9	69 ± 8	69 ± 11	72 ± 10	0.2941
Body Mass Index (kg/m ²)	23.3 ± 3.1	24.0 ± 2.7	23.4 ± 3.5	22.9 ± 2.8	0.2449
Hs-CRP (mg/L) [§]	1.40 (0.60, 3.00)	0.55 (0.33, 0.70)	1.60 (1.20, 2.20)	6.5 (4.03, 11.85)	<0.0001
BNP (pg/mL) [§]	59 (18, 177)	29 (15, 89)*	66 (16, 203)	102 (56, 479)	0.0009
8-isoprostane (pg/mg·cr)	166 ± 84	153 ± 73	174 ± 97	174 ± 81	0.3653
Lipid Status					
Total Cholesterol (mg/dL)	194 ± 39	194 ± 35	195 ± 41	195 ± 44	0.9867
Triglycerides (mg/dL)	133 ± 58	133 ± 58	137 ± 63	126 ± 51	0.7068
HDL-Cholesterol (mg/dL)	50 ± 14	54 ± 14*	49 ± 13	46 ± 14	0.0208
LDL-Cholesterol (mg/dL)	122 ± 34	117 ± 29	125 ± 37	129 ± 35	0.2837
Fasting Plasma Glucose (mg/dL)	114 ± 40	113 ± 28	114 ± 38	117 ± 58	0.9051
Hemoglobin (g/dL)	13.3 ± 1.7	13.4 ± 1.7	13.5 ± 1.7	13.0 ± 1.6	0.3537
Uric acid (mg/dL)	6.0 ± 1.5	5.9 ± 1.5	5.8 ± 1.4	6.4 ± 1.8	0.1886
eGFR (mL/min)	63 ± 25	65 ± 19	68 ± 31*	53 ± 21	0.0359
Previous MI (n (%))	56 (43)	25 (48)	19 (40)	12 (38)	0.5866
Previous PCI (n (%))	57 (43)	27 (52)	19 (40)	11 (34)	0.2495
Previous CABG (n (%))	10 (8)	4 (8)	2 (4)	4 (13)	0.4045
History of CHF (n (%))	33 (25)	8 (15)	12 (26)	13 (41)	0.0369
Coronary Risk Factors					
Hypertension (n (%))	95 (73)	42 (81)	30 (64)	23 (72)	0.1652
Diabetes mellitus (n (%))	35 (27)	18 (35)	9 (19)	8 (25)	0.2131
Smoking (n (%))	40 (31)	14 (27)	17 (36)	9 (28)	0.5773
Hyperlipidemia (n (%))	53 (40)	22 (42)	18 (38)	13 (41)	0.9206
Medications					
Aspirin (n (%))	102 (78)	42 (81)	38 (81)	22 (69)	0.3802
ACEI (n (%))	18 (14)	9 (17)	4 (9)	5 (16)	0.3976
ARB (n (%))	40 (31)	15 (29)	15 (32)	10 (31)	0.9417
Statin (n (%))	31 (24)	16 (31)	8 (17)	7 (22)	0.2642

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

Results

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 interquartile; 0.60 and 3.00) mg/L. Patient groups divided by hsCRP showed significant differences in plasma BNP, plasma HDL-cholesterol, and eGFR (*p* = 0.0009, *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).

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-isoprostane may correlate with coronary atherosclerosis and cardiovascular events⁽⁶⁻⁸⁾, but TBARS and 8-isoprostane 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

Fig. 1. Serum D-ROMs levels among the three groups.

There were significant differences in serum D-ROMs levels among the three groups ($p < 0.0001$ by ANOVA).

Mean and 95% confidential intervals of serum D-ROMs levels are shown.

method, hydroperoxides are converted into radicals that oxidize N,N-diethyl-para-phenyldiamine, which can be detected spectrophotometrically^{12, 13}, even in the clinic^{9, 14}. Serum levels of D-ROMs are between 200 to 300 U.CARR. in normal volunteers and are greater in subjects who smoke¹⁵, are on hemodialysis¹⁴, or who are hypertensive¹⁶.

We found elevated D-ROMs in patients with CAD and coronary risk factors. Interestingly, elevated levels of plasma 8-isoprostane, another marker of oxidative stress, are associated with the extent and severity of CAD^{17, 18}. In another study of 206 subjects, levels of TBARS correlated with disease severity as determined by angiography¹⁹; however, we did not

Table 2. Hemodynamic data and coronary angiography

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

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^{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 atherosclerosis²²⁾. These ROS initiate processes involved in atherogenesis through several important enzyme systems, including xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and nitric oxide synthase²³⁾. Moreover, CRP levels predict the risk for acute coronary syndrome²⁴⁾ and for future myocardial infarction and stroke in apparently healthy men⁴⁾. CRP is expressed in atherosclerotic lesions^{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 phosphatidylcholine²⁷⁾. 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 cells²⁸⁾; thus, CRP may be an inflammatory marker and stim-

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 $p<0.0001$).

ulate 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.

Table 3. Univariate and Multivariate Analysis of hsCRP

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

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^{2, 29)}, but with a lower odds ratio than other risk factors³⁰⁾; however, the combination of hsCRP and the TC/HDL-C ratio carries a greater projected risk³¹⁾, and cardiovascular event-free survival rates were lower in the high CRP and high LDL groups than in others³²⁾. Thus, the entire adult population does not require screening for hsCRP to assess cardiovascular risk⁵⁾, but in the future it may be part of a battery of tests for predicting risk⁵⁾. 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^{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) *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.

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