

Association of Oxidative DNA Damage and C-Reactive Protein in Women at Risk for Cardiovascular Disease

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Objective—The aim of the current study was to examine the relationship between clinical markers of inflammation and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an oxidative stress marker, in middle-aged women drawn from the HANDLS study, a longitudinal epidemiological study.

Methods and Results—We examined commonly assayed markers of inflammation, the DNA base adduct 8-oxodG, a marker of oxidative stress, and cardiovascular risk factors in a cohort of women matched on age and race in 3 groups (n=39 per group) who had low (<3 mg/L) high-sensitivity C-reactive protein (hsCRP), mid (>3–20 mg/L), and high (>20 mg/L) hsCRP. We found a significant relationship between hsCRP level and the oxidative stress marker, 8-oxodG. 8-oxodG was positively correlated with systolic blood pressure, pulse pressure, and interleukin-23. hsCRP was associated with obesity variables, high-density lipoprotein, serum insulin levels, interleukin-12p70 and intracellular adhesion molecule-1. Incubation of primary human endothelial cells with hsCRP generated reactive oxygen species in vitro. Furthermore, hsCRP specifically induced DNA base lesions, but not other forms of DNA damage, including single and double strand breaks.

Conclusion—These data suggest that in women 8-oxodG is associated with hsCRP and is independently related to select cardiovascular risk factors. Our data in women suggest that hsCRP may contribute to cardiovascular disease by increasing oxidative stress. (*Arterioscler Thromb Vasc Biol.* 2012;32:2776-2784.)

Key Words: 8-oxo-7,8-dihydro-2'-deoxyguanosine ■ DNA damage ■ oxidative stress ■ C-reactive protein
■ inflammation ■ women's health ■ cardiovascular disease

Accumulating evidence indicates that the levels of high-sensitivity C-reactive protein (hsCRP) are important for assessment of cardiovascular disease (CVD) risk and for the diagnosis and treatment of CVD. Recent data suggests that hsCRP is not only a marker of inflammation but may also contribute to the pathogenesis of vascular disease.¹⁻³ However, whether hsCRP plays a direct role in promoting atherosclerotic processes is still controversial and little is known about how hsCRP may elicit these effects.⁴ Nonetheless, important information has been obtained from correlating findings discovered from human populations to in vitro studies. For example, the proinflammatory cytokine interleukin (IL) 6 is known to induce hsCRP expression, and in women hsCRP levels correlate directly with IL-6.⁵⁻⁷ In addition, hsCRP has been shown to affect the expression of various proteins that are important for atherosclerosis and thrombosis and is present in atherosclerotic lesions.^{1-3,8}

Compelling data supports the idea that atherosclerosis is an inflammatory process. Furthermore, the coronary plaque environment is composed of multiple cell types, including macrophages and other inflammatory cells that secrete cytokines and reactive oxygen species (ROS) that drive inflammation and oxidative stress.⁹ ROS generation is an important signaling mechanism in cells.¹⁰ However, it can be detrimental to cellular homeostasis

by leading to DNA damage, which if left unrepaired, can lead to mutations that cause disease. Persuasive evidence suggests that oxidative stress induced DNA damage and genetic alterations may contribute to atherosclerosis.^{11,12} Therefore, it is likely that both oxidative stress and inflammatory markers may suggest disease burden or risk. Although extensive research has focused on this link, it remains to be determined which markers can be used clinically to indicate both inflammatory and oxidative stress states.¹³

Levels of the DNA damage base lesion, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), can be associated with increased oxidative stress as high levels of serum 8-oxodG can be detected in patients with systemic lupus erythematosus, Parkinson disease, end-stage renal disease, and type 2 diabetes mellitus,¹⁴⁻¹⁷ and in urine and from specific tissues of various diseases.¹⁸ 8-oxodG has also been found to be increased in atherosclerosis plaques^{11,19,20} and in lymphocytes from men with premature coronary heart disease mortality.²¹ Although hsCRP and serum 8-oxodG have been found to correlate in patients with end-stage renal disease,¹⁷ little is known about the relationship between these 2 markers, particularly in community-dwelling participants.

Here, we have focused on the association between inflammation and oxidative stress by examining commonly assayed

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inflammatory markers and the DNA base adduct 8-oxodG, as a marker of oxidative stress in a cohort of middle-aged women with low, mid-range, and high hsCRP values who are also participants in the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study, an interdisciplinary epidemiological study of health disparities in the city of Baltimore. Although traditionally in clinical settings hsCRP levels >10 mg/L have been associated with acute illness, infections, or autoimmune disorders but not CVD risk; data from the Women's Health study showed that hsCRP values remain predictive for cardiovascular events for the entire range of values.^{22,23} In fact, women in the Women's Health Study with hsCRP values >10 mg/L were considered to be a very high risk group and those with hsCRP values \geq 20 mg/L were observed to be at exceptionally elevated risk for vascular disease. Moreover, women have higher levels of hsCRP than men^{24,25} and are particularly vulnerable to CVD. CVD has been cited as the challenge of the middle years for women because CVD is the primary cause of death among women in the United States, and currently, more women die from CVD than men in the United States.²⁶ We found that serum hsCRP levels correlate significantly with 8-oxodG. We have further investigated the connection between hsCRP and 8-oxodG and found that hsCRP induces ROS and DNA base lesions in vitro. Our findings suggest that hsCRP production may enhance inflammatory processes, such as atherosclerosis and thrombosis, in part, by increasing oxidative stress and inducing DNA damage.

Materials and Methods

Participants

We conducted a nested-cohort study comparing inflammatory markers with the oxidative stress marker 8-oxodG on women drawn from the (HANDLS) study of the National Institute on Aging Intramural Research Program,²⁷ approved by the MedStar Health Research Institute Institutional Review Board. HANDLS is an area probability sample of Baltimore City based on the 2000 census data, and this study examines the effects of race (whites and African Americans) and socioeconomic status (below or above 125% of the Federal poverty level) on health outcomes in a cohort of urban adults aged initially between 30 to 64. All the selected women gave written consent to store serum, had available serum for examination, and had completed HANDLS baseline assessment. We matched 3 groups of women (39 per group) who had low (<3 mg/L) hsCRP, mid (>3–20 mg/L), and high (>20 mg/L) hsCRP based on age and race. Eighty-six women in the total HANDLS study cohort had hsCRP values >20.

Physical Measurements and Laboratory Assays

Blood pressure was averaged for assessments in both arms while seated after a 5 minute rest. Body mass index (weight [kg]/height [m²]) was computed from measured height and weight. Clinical conditions were recorded based on a structured medical history interview and a physical examination. We obtained fasting blood samples the serum from which was assayed by Quest Diagnostics (Nichols Institute, Chantilly, VA) or stored at -80°C . Fasting glucose, insulin, cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), creatinine, lactate dehydrogenase (LDH), and hsCRP were measured at Quest Diagnostics. Various cytokines and inflammatory markers were measured in serum using Searchlight protein arrays from Aushon Biosystems (Billerica, MA).

Cell Lines and Reagents

Human umbilical vein endothelial cells ([HUVECs]; Clonetics) were purchased from Lonza and were maintained in EBM-2 media supplemented with EGM-2 SingleQuots (Lonza). Highly purified human recombinant CRP without sodium azide and free of endotoxins was specially obtained from Trichem Resources Inc.

For Figure 1C, HUVECs were incubated in a 1:10 dilution of growth media for 18 hours in the presence or absence of hsCRP (25 $\mu\text{g}/\text{mL}$). Cells were lysed in sample buffer, separated by SDS-PAGE and immunoblotted with anti-p22^{phox} (CS9; Santa Cruz), anti-Nox1 (Sigma-Aldrich), or anti-actin antibodies (I-19; Santa Cruz) as a protein loading control.

8-oxodG ELISA

For 8-oxodG detection, we used the highly sensitive 8-OHdG (8-oxodG) ELISA Check kit from the Japan Institute for the Control of Aging (purchased from Genox Inc, Baltimore, MD). The specificity of the monoclonal antibody used in the ELISA assay has been previously reported.²⁸ As suggested by the manufacturer, serum samples (0.3 mL) were centrifuged at 15000g for 60 minutes at 4°C through a Vivaspin ultrafiltration spin column (10000 molecular weight cut-off; Sartorius-Stedim Biotech). The serum filtrate (50 μL) was used directly for the ELISA assay or frozen at -80°C . The ELISA assay was performed blind according to manufacturer's instructions and was repeated in 2 independent experiments. There was a significant degree of repeatability in the 2 8-oxodG assays (intraclass correlation = 0.45; $P < 0.001$) and the average 8-oxodG value from the 2 experiments was used for further analysis.

Immunofluorescence

To monitor superoxide production, HUVECs were plated on 4-well chamber slides, and 18 hours later cells were incubated in serum-free media with either the superoxide indicator dihydroethidium (3 $\mu\text{mol}/\text{L}$; Molecular Probes) or MitoSOX (3 $\mu\text{mol}/\text{L}$; Molecular Probes) to detect mitochondrial superoxide. In addition, either 25 $\mu\text{g}/\text{mL}$ hsCRP, 40 $\mu\text{mol}/\text{L}$ pyranocyanin (to induce superoxide; Enzo Life Sciences), or 10 $\mu\text{mol}/\text{L}$ Antimycin A (to induce mitochondrial superoxide; Sigma-Aldrich) was added to the wells and incubated for 30 minutes. Fluorescent images of superoxide or mitochondrial superoxide and phase images were taken on a Zeiss Observer D1 microscope with an AxioCam1Cc1 camera at a set exposure time.

HUVECs were treated for 30 minutes with 10 $\mu\text{mol}/\text{L}$ menadione, 25 $\mu\text{g}/\text{mL}$ hsCRP (or the indicated hsCRP concentrations), 5 mmol/L N-acetyl-L-cysteine ([NAC]; Enzo Life Sciences) or hsCRP, and NAC in serum-free media. NAC was added 30 minutes before the addition of CRP (for the NAC+CRP sample). Staining for 8-oxo-7,8-dihydroguanine (8-oxoG) and 4',6-diamidino-2-phenylindole (DAPI) was performed as previously described.²⁹ Pictures were taken using a Zeiss Observer D1 microscope with an AxioCam1Cc1 camera at a set exposure time, and the fluorescence intensity of 8-oxoG-stained nuclei were quantified from duplicate coverslips using AxioVision Rel 4.7 software. The histograms in Figure 2B and 2C represent the mean fluorescence intensity from 3 independent experiments.

Statistical Analyses

Thirty-seven women per group had a statistical power of 80% to detect a difference at least as large as 0.33 SD for matched comparisons between groups with $P < 0.05$ and power $(1-\beta) = 0.8$. We used mixed-model regressions to examine group differences (Table 1) and the effects on oxidative stress after adjusting for covariates. Categorical measures were examined with logistic regression. We included interactions in regression analyses only after they were significant after a backward elimination procedure for nonsignificant effects. We applied a \log_{10} transform to hsCRP because the distribution was skewed. We used R to perform all analyses.³⁰

Table 1. Means and SD With Separate Comparisons of Middle and High hsCRP Groups With the Low hsCRP Group on Age, Anthropometrics, Blood Pressure, Pulse Pressure, Clinical Laboratory Assays, and Inflammatory Markers

	hsCRP Groups (n=39 Per Group)							
	Low (≤ 3 mg/L)		Mid (>3 – 20 mg/L)			High (>20 mg/L)		
	Mean	SD	Mean	SD	<i>P</i> Value	Mean	SD	<i>P</i> Value
Age, y	49.77	8.81	49.77	8.81	1	49.77	8.81	1
hsCRP, log ₁₀ (hsCRP) in mg/L	-0.46	1.02	1.87	0.51	0.00**	3.4	0.43	0.00**
8-oxodG	0.16	0.04	0.17	0.04	0.13	0.18	0.04	0.00**
BMI	26.8	5.13	34.74	9	0.00**	39.63	11.94	0.00**
Weight, kg	71.72	14.64	93.23	23.74	0.00**	103.03	32.95	0.00**
Waist size, cm	92.39	13.48	106.88	17.43	0.00**	114.97	23.44	0.00**
Hip size, cm	102.68	13.29	115.79	17.64	0.00**	124.35	20.96	0.00**
Waist-hip ratio	0.9	0.08	0.92	0.07	0.21	0.92	0.08	0.44
Diastolic BP, mm Hg	71.49	11.68	75.08	11.28	0.14	69.89	8.81	0.95
Systolic BP, mm Hg	118.59	18.24	126.42	18.49	0.05	125.49	20.4	0.08
Pulse pressure	47.11	12.95	51.34	14.35	0.13	55.6	15.81	0.02*
Total cholesterol, mg/dL	187.18	34.86	196.05	43.38	0.33	182.21	45.9	0.64
LDL, mg/dL	104.07	34.74	115.51	37.43	0.17	108.89	43.83	0.53
HDL, mg/dL	60.26	16.09	52.54	13.97	0.02*	47.74	13.32	0.00**
Triglycerides, mg/dL	114.21	81.17	139.95	74.63	0.08	127.82	61.26	0.34
Creatinine, mg/dL	0.96	0.87	0.87	0.19	0.39	0.84	0.22	0.33
Glucose, mg/dL	94.18	10.14	100.87	17.19	0.4	150.59	80.97	0.00**
Insulin, μ U/mL	10.33	6.87	12.31	8.75	0.35	17.23	12.69	0.01*
LDH, U/L	160.4	32.25	178.15	36.33	0.05	163.08	34.79	0.68
eGFR	86.3	18.53	85.9	21.54	0.97	90.9	25.37	0.26
IL-1, pg/mL	5.82	19.47	1.22	2.46	0.26	2.9	6.77	0.34
IL-6, pg/mL	8.47	11.89	5.94	4.29	0.27	15.78	15.18	0.03*
IL-8, pg/mL	17.06	11.41	8.81	7.87	0.00**	18.38	12.27	0.75
IL-10, pg/mL	3.02	4.38	0.74	0.37	0.01**	2.19	1.12	0.52
IL-12p70, pg/mL	1.54	3.12	0.62	0.75	0.04*	0.74	0.98	0.07
IL-18, pg/mL	146.13	63.4	221.25	139.07	0.00**	171.45	115.41	0.32
IL-23, pg/mL	1636.76	7936.71	594.73	1077.08	0.39	776.51	2925.65	0.56
RAGE, pg/mL	1014.3	502.16	374.73	468	0.00**	1100.76	575.73	0.57
ICAM-1, pg/mL	371 586.96	133 792.62	532 839.13	183 161.02	0.00**	479 242.83	189 260.41	0.01
VCAM-1, pg/mL	1 680 988.5	1 325 971.5	1 188 016	665 613.58	0.07	1 290 350.9	459 042.68	0.11
MCP-1, pg/mL	919.65	461.14	700.59	558.92	0.11	979.58	737.66	0.67
TNF- α , pg/mL	39.29	122.24	5.98	15.34	0.2	22.55	55.16	0.49

hsCRP indicates high-sensitivity C-reactive protein; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; eGFR, estimated glomerular filtration rate; IL, interleukin; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion protein-1; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor- α .

* $P < 0.05$; ** $P < 0.01$.

Results

Relationship Between hsCRP and Cardiovascular Risk Factors

To examine the relationship between hsCRP levels in women and other variables, we studied a cohort of women with low (<3 mg/L), mid (>3 – 20 mg/L), or high hsCRP levels (>20 mg/L). Each group contained 39 women and the groups were age (mean age, 49.7 ± 8.1 years) and race (19 whites, 20 African Americans) matched. There were no differences between low, mid, and high hsCRP groups on the 2 matching

variables. Women in the mid- and high hsCRP groups were significantly heavier than women in the low hsCRP group, had larger waist and hip sizes, and had greater body mass index (Table 1). Women in the mid-hsCRP group had significantly higher systolic blood pressure than women in the low hsCRP, but women in the high hsCRP group had greater pulse pressure suggesting that in this group increased hsCRP is associated with less compliant arteries at greater levels of inflammation. Women in the mid and high hsCRP group also had lower HDL levels and higher levels of fasting glucose and insulin.

Given the fact that hsCRP is an acute phase marker of inflammation and that atherosclerosis and atherothrombotic events are inflammatory processes, we examined the differences in the inflammatory profile of women based on the hsCRP level. Surprisingly, there were few consistent significant differences in most of the inflammatory markers by hsCRP group (Table 1). Consistent with other studies, we found that IL-6 and intracellular adhesion molecule-1 (ICAM-1) levels were higher in women with high hsCRP.^{5,31} Compared with women in the low hsCRP group, women in the mid group had greater levels of IL-18 and ICAM-1 but lower levels of IL-8, IL-10, IL-12p70, and receptor for advanced glycation endproducts.

In addition to the inflammatory markers, we also examined whether hsCRP was associated with the oxidative stress marker, 8-oxodG. Women in the mid-hsCRP group had higher levels of 8-oxodG than the low hsCRP group, and women in the high hsCRP group had significantly greater 8-oxodG levels than the low hsCRP group (Table 1).

Association of hsCRP With Inflammation-Related Conditions and Diagnoses

Although participants in the HANDLS study are community dwelling, several of the individuals had preexisting chronic medical illnesses. Because some of these conditions are associated with inflammation and oxidative stress, we examined whether hsCRP levels were affected by the presence of these conditions. Inflammatory conditions and diagnoses were more prevalent in both the mid and high hsCRP groups than the low hsCRP group (Table 2). Those in the high hsCRP group had greater rates of self-reported asthma (OR=3.63; 95% CI=1.17–12.87); diabetes mellitus (OR=10.29; 95%

CI=3.30–39.80); and hypertension (OR=3.49; 95% CI=1.36–9.40) but not rheumatoid arthritis, osteoarthritis, psoriasis, stroke, rates of cigarette smoking or statin use, coronary heart disease, metabolic syndrome, or positive serology for hepatitis B and hepatitis C. Those in the mid-hsCRP group had greater rates of metabolic syndrome (OR=2.78; 95% CI=1.09–7.40) and hypertension (OR=2.63; 95% CI =1.05–6.78), but not rheumatoid arthritis, osteoarthritis, psoriasis, stroke, rates of cigarette smoking or statin use, coronary heart disease, diabetes mellitus, or positive serology for hepatitis B and hepatitis C.

Association of 8-oxodG With Age, hsCRP, Anthropometrics, and Inflammation-Related Markers

8-oxodG was associated with age, inflammatory markers, anthropometric measures in the complete sample, but the pattern of associations differed between the high, mid, and low hsCRP groups. In the complete sample, 8-oxodG was associated with age ($r=0.33$; $P<0.001$), hsCRP ($r=0.21$; $P=0.02$), body mass index ($r=0.20$; $P=0.03$), hip size ($r=0.19$; $P=.04$), systolic blood pressure ($r=0.32$; $P<0.001$), pulse pressure ($r=0.33$; $P<0.001$), and IL-23 ($r=0.24$; $P=.02$). In the low hsCRP group, 8-oxodG was associated with age ($r=0.32$; $P=0.04$), diastolic blood pressure ($r=0.34$; $P=0.04$), systolic blood pressure ($r=0.53$; $P<0.001$), pulse pressure ($r=0.45$; $P=0.01$), creatinine ($r=0.39$; $P=0.01$), LDH ($r=0.38$; $P=0.04$), and IL-23 ($r=0.40$; $P=0.01$). In the mid-hsCRP group only age ($r=0.36$; $P=0.02$) was associated significantly with 8-oxodG, and in the high hsCRP group only age ($r=0.36$; $P=0.03$) and LDH ($r=0.08$; $P=0.04$) were associated significantly with 8-oxodG.

Table 2. Rates of Inflammatory-Related Conditions and Diagnoses With Separate Comparisons of Middle and High hsCRP Groups With the Low hsCRP Group

	hsCRP Groups (n=39 Per Group)						
	Low (≤3 mg/L)		Mid (>3–20 mg/L)		High (>20 mg/L)		
	Rate	Rate	OR	P Value	Rate	OR	P Value
Asthma (hx)	0.18	0.229	1.36	0.62	0.44	3.63	0.03*
Rheumatoid arthritis (hx)	0.13	0.054	0.37	0.27	0.03	0.22	0.18
Osteoarthritis (hx)	0.21	0.216	1.06	0.92	0.42	2.77	0.08
Psoriasis (hx)	0.04	0.029	0.76	0.85	0.08	2.17	0.53
Stroke (hx)	0.03	0.056	1.94	0.59	0.03	0.97	0.98
Current cigarette smoker	0.47	0.47	1	1	0.33	0.56	0.23
Statin use	0.19	0.16	0.78	0.68	0.33	2.07	0.19
Coronary heart disease (hx)	0.19	0.211	1.1	0.86	0.39	2.64	0.07
Metabolic syndrome [†]	0.36	0.611	2.78	0.03*	0.7	2.12	0.12
Diabetes mellitus [‡]	0.1	0.256	3.02	0.08	0.54	10.29	0.00**
Hypertension [§]	0.39	0.632	2.63	0.04*	0.69	3.48	0.01*
Hepatitis B (assay)	0.05	0	0	0.99	0.03	0.51	0.59
Hepatitis C (assay)	0.21	0.132	0.57	0.36	0.08	0.34	0.13

hx indicates by structured medical history; OR, odds ratio.

* $P<0.05$; ** $P<0.01$.

[†]ATP-III⁴⁸.

[‡]Fasting glucose ≥140 mg/dL, medication for diabetes mellitus, or self-report.

[§]Measured blood pressure ≥140/90, antihypertensive medication, or self-report.

8-oxodG Levels Are Associated With Cardiovascular Risk Factors

We examined the association of 8-oxodG, hsCRP, race, and poverty status with anthropometric measures, blood pressure, clinical laboratory assays, and inflammatory markers (Table 3). None of the 4-way, 3-way, or 2-way interactions were significant, and they were removed by backward elimination. Adjusting for hsCRP, race, and poverty status, 8-oxodG was associated with systolic blood pressure and pulse pressure but not with anthropometric measures, clinical laboratory assays, or inflammatory markers. Adjusting for 8-oxodG, race, and poverty status, hsCRP was associated with body mass index, weight, waist and hip size, waist-hip ratio, HDL, fasting glucose, insulin, IL-12p70, and ICAM-1. Adjusting for 8-oxodG, hsCRP, and poverty status, race was associated with systolic blood pressure,

pulse pressure, LDL, triglycerides, receptor for advanced glycation endproducts, ICAM-1, vascular cell adhesion protein-1, and monocyte chemoattractant protein-1. Adjusting for 8-oxodG, hsCRP, and race, poverty status was associated with weight, hip size, total cholesterol, LDL, HDL, and ICAM-1.

CRP Increased ROS Production and DNA Base Lesions

The strong relationship between 8-oxodG levels and hsCRP and the fact that recent data suggest that CRP may contribute to the inflammatory disease process led us to investigate whether hsCRP itself can increase oxidative stress and potentially induce DNA damage. We found that hsCRP induces ROS production in HUVECs (Figure I in the online-only Data Supplement), consistent with other reports.^{8,32} In vascular cells,

Table 3. Regression of Anthropometrics, Blood Pressure, Pulse Pressure Clinical Laboratory Assays, and Inflammatory Markers on 8-oxodG, hsCRP, race, and Poverty Status With Separate Comparisons of Middle and High hsCRP Groups With the Low hsCRP Group adjusting for Matching

	8-oxodG		hsCRP, log ₁₀ (hsCRP) in mg/L		Race		Poverty Status	
	Coefficient	P Value	Coefficient	P Value	Coefficient	P Value	Coefficient	P Value
BMI	26.94	0.22	2.99	0.00**	-0.79	0.63	-2.83	0.08
Weight, kg	58.41	0.32	7.63	0.00**	-3.92	0.36	-9.82	0.02*
Waist size, cm	39.96	0.36	5.71	0.00**	-5.27	0.1	-5.64	0.09
Hip size, cm	56.02	0.18	4.91	0.00**	-5.66	0.07	-8.09	0.01*
Waist-hip ratio	-0.11	0.55	0.01	0.03*	0	0.96	0.02	0.27
Diastolic BP, mm Hg	34.34	0.19	-0.37	0.6	0.43	0.81	-3.24	0.1
Systolic BP, mm Hg	166.18	0.00**	0.7	0.49	9.04	0.01*	-1.25	0.72
Pulse pressure	127.51	0.00**	0.68	0.36	8.47	0.00**	-0.2	0.94
Total cholesterol, mg/dL	79.03	0.42	-0.51	0.82	11.73	0.09	-25.07	0.00**
LDL mg/dL	38.98	0.67	1.25	0.56	16.77	0.01*	-19.65	0.00**
HDL, mg/dL	17.2	0.63	-3.38	0.00**	2.48	0.35	-5.62	0.04*
Triglycerides, mg/dL	-94.78	0.6	5.65	0.14	-34.2	0.01*	2.31	0.86
Creatinine, mg/dL	-0.53	0.32	0	0.86	0.06	0.1	0.02	0.69
Glucose, mg/dL	30.62	0.75	4.74	0.07	-0.85	0.91	6.71	0.36
Insulin, μ IU/mL	-5.87	0.81	1.58	0.00**	-0.68	0.7	1.44	0.42
LDH, U/L	-6.58	0.95	3.4	0.14	2.37	0.77	-8.45	0.29
eGFR	-11.8	0.83	0.92	0.43	6.08	0.14	0.02	0.99
IL-1, pg/mL	3.4	0.92	-1.21	0.1	2.74	0.32	2.39	0.4
IL-6, pg/mL	2.85	0.92	0.85	0.22	0.55	0.78	1.14	0.59
IL-8, pg/mL	14.27	0.61	-1.18	0.06	-2.51	0.19	3.48	0.08
IL-10, pg/mL	-0.13	0.98	0.03	0.82	-0.28	0.49	-0.12	0.79
IL-12p70, pg/mL	3.53	0.48	-0.23	0.03*	0.02	0.96	-0.13	0.74
IL-18, pg/mL	251.75	0.38	8.88	0.15	-33.7	0.11	33.86	0.11
IL-23, pg/mL	3522.61	0.4	229.53	0.29	44.22	0.89	79.39	0.83
RAGE, pg/mL	139.37	0.92	-48.41	0.1	-209.51	0.03*	2.51	0.98
ICAM-1, pg/mL	280653.5	0.52	31631.42	0.00**	-71777.8	0.03*	63749.37	0.05
VCAM-1, pg/mL	1152114	0.48	-9018.53	0.86	-339555	0.00**	147345.7	0.26
MCP-1, pg/mL	1488.47	0.31	9.58	0.77	-373.5	0.00**	67.42	0.54
TNF- α , pg/mL	183.58	0.39	-6.85	0.19	10.69	0.52	30.05	0.08

8-oxodG indicates 8-oxo-7,8-dihydro-2'-deoxyguanosine; hsCRP, high-sensitivity C-reactive protein; BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; eGFR, estimated glomerular filtration rate; IL, interleukin; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion protein-1; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor- α ; RAGE, receptor for advanced glycation endproducts.

* $P < 0.05$; ** $P < 0.01$.

ROS is mainly produced by the NADPH oxidase enzyme complex.^{33–35} Therefore, we examined whether longer exposure (18 hours) to hsCRP changes expression of the different NADPH oxidase (Nox) isoforms that are known to be important for vascular generation of ROS. We found that CRP increases the protein level of Nox1 but did not affect expression of Nox2, Nox4, and Nox5 (Figure 1 and Figure II in the online-only Data Supplement). Similar to previous findings, we found that hsCRP upregulated p22^{phox} protein expression, a required subunit of NADPH oxidase complex.¹ We also examined various antioxidant enzymes and found a modest upregulation of the cytoplasmic antioxidant enzyme, glutathione peroxidase (Figure II in the online-only Data Supplement). However, hsCRP did not change the protein level of catalase, superoxide dismutase-1, or superoxide dismutase-2 (Figure II in the online-only Data Supplement). Because NADPH oxidase complexes generate superoxide and we observed an upregulation of Nox1 and p22^{phox}, we examined whether we could detect superoxide in response to hsCRP. The well-known superoxide producer pyranocyanin and hsCRP both induced intracellular superoxide (Figure 1B). In contrast to antimycin A, which is known to generate mitochondrial superoxide, we did not detect mitochondrial superoxide induction by hsCRP (Figure 1C). Taken together, these data suggest that hsCRP primarily induces intracellular ROS rather than mitochondrial ROS.

To examine whether hsCRP induces DNA damage, we immunostained HUVECs with antibodies against the DNA base lesion, 8-oxoG, an analog of 8-oxodG that can be easily detected in tissue culture cells by immunostaining.^{29,36,37} Untreated HUVECs have a background level of 8-oxoG, which is consistent with previous reports.^{29,36,37} However, treatment with clinically relevant doses of hsCRP increased the level of 8-oxoG in a dose-dependent manner (Figure 2A–2C). Furthermore, measurement of the fluorescent intensity of staining revealed that hsCRP increases 8-oxoG levels similar to the DNA-damaging agent menadione (Figure 2B). To determine whether hsCRP induction of ROS contributes to 8-oxoG formation, we treated HUVECs with hsCRP in the presence of the antioxidant, NAC, to inhibit ROS (Figure 2). The level of 8-oxoG was similar to untreated cells when cells were incubated with NAC alone or in combination with hsCRP, indicating that ROS is important for CRP-induced base damage.

We employed the alkaline comet assay to assess whether, in addition to 8-oxoG, hsCRP can induce other forms of DNA damage. Under these conditions the comet assay measures alkaline sensitive sites that include single strand breaks (SSBs), alkaline labile sites, and transient repair sites. Increased alkaline sensitive sites were observed in cells treated with H₂O₂, but not in HUVECs treated with hsCRP for 30 or 60 minutes (Figure IIIA in the online-only Data Supplement). In addition, we examined whether hsCRP influences the level of double strand breaks (DSBs) by staining for phosphorylated H2AX (referred to as γ -H2AX), a marker of DSBs. As expected, staurosporine treatment increased γ -H2AX foci formation (Figure IIIB in the online-only Data Supplement). However, hsCRP stimulation did not affect the levels γ -H2AX, suggesting that hsCRP does not substantially induce DSBs (Figure IIIB in the online-only Data Supplement).

We also examined whether hsCRP can affect lipid peroxidation, another marker of oxidative stress. As measurement

of malondialdehyde (MDA) has been used as a common indicator of lipid peroxidation, we analyzed MDA levels in HUVECs treated with hsCRP. hsCRP significantly increased the levels of MDA compared with untreated cells (Figure IV in the online-only Data Supplement), suggesting that hsCRP can also induce products of lipid peroxidation.

Discussion

To gain a better understanding about the role of inflammation and oxidative stress in women at high risk for CVD, we examined a cohort of women in the HANDLS study based on 3 categories of hsCRP levels, low (<3 mg/L), mid (>3–20 mg/L), and high (>20 mg/L), as this represents women at low, high, and extremely elevated risk for CVD. Each of these groups contained 39 women and were age and race matched. In this study, we found that several clinical risk factors for CVD were associated with high circulating levels of hsCRP, including, obesity-related parameters, HDL, glucose, insulin, IL-12p70, and ICAM-1. As could be expected, we observed that metabolic syndrome and hypertension were higher in women with mid-range hsCRP values. Diabetes mellitus, asthma, and hypertension were all associated with high levels of hsCRP. However, hsCRP levels were also associated with the oxidative stress marker, 8-oxodG. These results lend credence to the idea that there may be close relationships between inflammation and certain forms of oxidative stress. However, a limited number of studies have investigated the relationship between hsCRP and serum 8-oxodG, especially in cohorts of community-dwelling individuals not stratified by disease.

8-oxodG is an important oxidative stress marker because it is one of the most abundant oxidative base lesions and is also highly mutagenic.³⁸ The contribution of DNA mutagenesis to CVD is still unclear. However, emerging data indicate that DNA damage and other genetic alterations may play a pivotal role in the pathogenesis of atherosclerosis.^{11,12} Specifically, increased levels of various DNA adducts, including 8-oxodG, have been found in atherosclerotic lesions.^{19,20,39,40} In addition, 8-oxodG accumulates in hypertensive aged rats⁴¹ and may be related to serum levels of antibodies against oxidized LDL, which is important because oxidation of LDL and the generation of antibodies against oxidized LDL are implicated in the progression of atherosclerosis.⁴² In this report, additional evidence suggests that in humans 8-oxodG levels can be reduced on treatment with fluvastatin, indicating that cholesterol lowering therapies may reduce oxidative stress burden and DNA damage levels. Similarly, decreased levels of urinary 8-oxodG and hsCRP were also observed after treatment with rosuvastatin, as well as calcium-blocking agents, in hypertensive patients.^{43–45}

These data and the fact that circulating lymphocytes from patients with atherosclerosis have higher levels of 8-oxodG than control subjects, and 8-oxodG accumulation in lymphocyte DNA is associated with higher rates of death from premature coronary artery disease,^{11,21,46} led us to examine the level of 8-oxodG in the serum rather than urinary 8-oxodG. Although a large body of work has identified urinary 8-oxodG as an informative oxidative stress marker,^{18,47} detection of this bio-specimen relies on careful complete collection and storage of 24-hour urine, which may be difficult to obtain. Examination of 8-oxodG in the serum has been widely used to compare 8-oxodG in normal and diseased patients.^{14–17} It should be noted that we

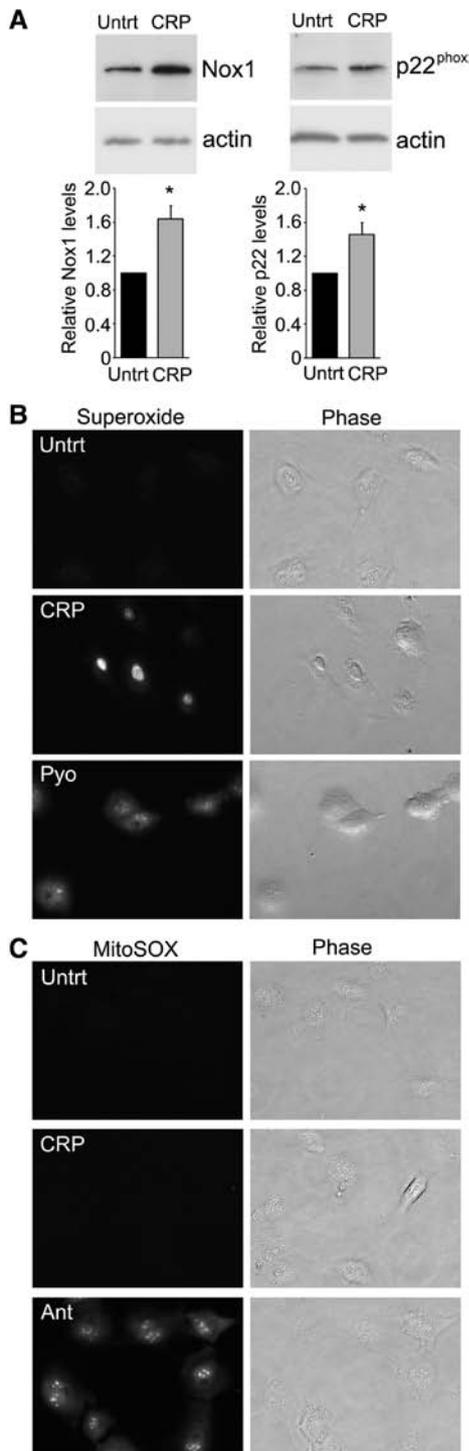


Figure 1. High-sensitivity C-reactive protein (hsCRP) induces superoxide in human umbilical vein endothelial cells (HUVECs). **A**, HUVECs were untreated or treated for 18 hours with hsCRP (25 μ g/mL), lysed and probed with anti-p22^{phox}, anti-NADPH oxidase 1 (Nox1) or anti-actin antibodies as a loading control. Relative levels of p22^{phox} or Nox1 were quantified from immunoblots and normalized to actin control. The histograms represent the normalized mean from 4 independent experiments +SEM. * P <0.05 by Student t test. **B** and **C**, HUVECs were incubated with either the superoxide detector dihydroethidium (**B**) or MitoSOX (**C**) to detect mitochondrial superoxide in serum-free media containing either 25 μ g/mL hsCRP, 40 μ mol/L pyranocyanin (Pyo) or 10 μ mol/L antimycin A (Ant). Untrt indicates mock treated. Representative images are shown and similar results were obtained in 3 independent experiments.

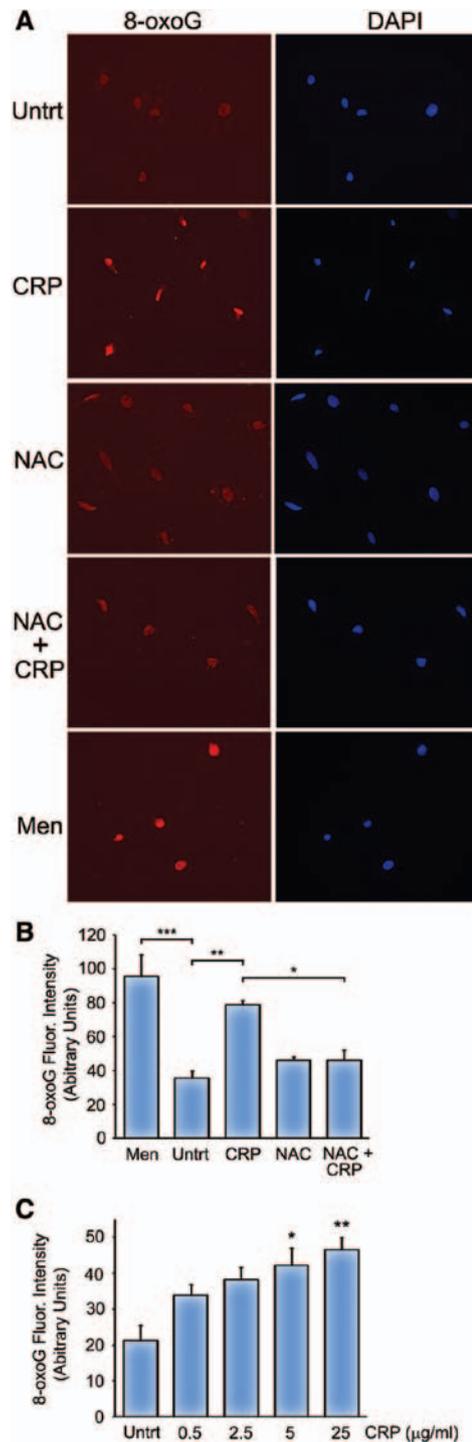


Figure 2. High-sensitivity C-reactive protein (hsCRP) induces 8-oxo-7,8-dihydro-guanine (8-oxoG) in HUVECs. **A**, HUVECs were mock treated (Untrt) or treated for 30 minutes with 10 μ mol/L menadione (Men), 25 μ g/mL hsCRP, 5 mmol/L N-Acetyl-L-cysteine (NAC) or hsCRP and NAC in serum-free media. NAC was added 30 minutes before the addition of CRP. HUVECs were stained with anti-8-oxoG antibodies (left) and 4,6-diamidino-2-phenylindole (DAPI; right). **B**, 8-oxoG fluorescent intensity was calculated as described in the Materials and Methods. **C**, 8-oxoG fluorescent intensity was calculated from HUVECs that were mock treated or treated with the indicated concentrations of hsCRP for 30 minutes and subsequently stained with anti-8-oxoG antibodies. The histograms represent the mean of 3 independent experiments +SEM. * P <0.05, ** P <0.01, *** P <0.001 for the indicated comparisons using 1-way ANOVA and Tukey's post hoc test.

used an ELISA assay to measure 8-oxodG levels. In most cases there is a consensus between chromatographic and ELISA measurements of urinary 8-oxodG; however, it is observed that higher levels of urinary 8-oxodG are obtained with the ELISA kit,^{47,48} although it remains to be determined whether this is also the case for serum levels of 8-oxodG. Nevertheless, our results show that 8-oxodG levels in the serum increase with age, which is consistent with data in the literature that this lesion and other biomarkers of oxidative stress increase with age.^{18,49}

Importantly, we observed that 8-oxodG levels are associated with clinical cardiovascular risk factors, such as age, hsCRP, body mass index, hip circumference, systolic blood pressure and pulse pressure, and IL-23 levels. Interestingly, 8-oxodG correlates with creatinine levels only in the low hsCRP group and with LDH in both the low and high hsCRP groups (but not the overall group or in the mid group), although in opposite ways. These relationships are interesting and suggest that 8-oxodG and hsCRP status may influence the levels of these clinical markers, and will be important to explore further in the future. 8-oxodG levels were significantly higher in participants who had osteoarthritis or were HIV positive, which is consistent with the fact that these diseases are associated with high levels of inflammation and oxidative stress.

Surprisingly, we found a strong association of 8-oxodG levels with systolic blood pressure and with pulse pressure, independent of hsCRP level. These data indicate that although there is a strong relationship between hsCRP and 8-oxodG, these markers may give an indication of different aspects of inflammation. For example, in our cohort of women, hsCRP may reflect the inflammatory status related to metabolic factors, whereas, 8-oxodG levels may give a better indication of the properties of blood vessels and vascular health. We also found that race was associated with systolic and pulse pressure, LDL, triglycerides, receptor for advanced glycation endproducts, ICAM-1, vascular cell adhesion protein 1, and monocyte chemoattractant protein-1. Poverty status was related to obesity parameters, cholesterol, LDL, HDL, LDH, and ICAM-1. It is interesting to speculate that some of the associations that we observe may contribute to the health disparities related to race and poverty status.

Increased levels of 8-oxodG with high hsCRP could be due to several reasons. First, our *in vitro* data demonstrate that hsCRP can increase the amount of ROS and induce the DNA base lesion, 8-oxoG. These data are consistent with previous reports that treatment of human endothelial and smooth muscle cells with hsCRP *in vitro* generates ROS^{8,32} and may further potentiate ROS signaling by increasing the levels of Nox1 and the p22^{phox} subunit of the NADPH oxidase complex, a primary source of ROS in cells.¹ In particular, increased expression and activation of Nox1 has been shown to promote vascular pathologies, including hypertension, atherosclerosis, and restenosis.^{33–35} Therefore, we can speculate that *in vivo* hsCRP may also induce 8-oxodG formation. Second, inflammatory cells are recruited to atherosclerotic plaques where they produce ROS and other inflammatory cytokines that can promote both inflammation and oxidative stress, leading to increased DNA damage. It will be interesting to examine in the future whether hsCRP induces ROS and increases Nox1 and p22^{phox} expression in other cell types other than endothelial cells. In addition, other

cardiovascular risk factors, such as obesity, hyperlipidemia, and hypertension, that have previously been shown to increase oxidative stress could also contribute to higher levels of 8-oxodG. Environmental factors and lifestyle could also affect levels of 8-oxodG. Furthermore, given our findings that hsCRP can increase MDA levels *in vitro*, hsCRP could also affect 8-oxodG levels *in vivo* by increasing MDA or other lipid peroxidation products that may in turn potentiate oxidative stress-induced DNA damage. It is interesting that we found that hsCRP did not induce other forms of DNA damage, including SSBs and DSBs. However, we cannot exclude that prolonged exposure to hsCRP and the increased levels of inflammation and oxidative stress associated with vascular diseases may induce 8-oxodG, as well as, SSBs and DSBs *in vivo*.

In conclusion, in our cohort of women we found that 8-oxodG and hsCRP are independently related to several cardiovascular risk factors. The present analyses were limited by a relatively small sample of participants. However, we had sufficient power to detect our anticipated effects and the sample size must be tempered by the complexity and manual labor involved in this assay. Future research lies in elucidating whether this relationship can be generalized in a larger cohort, as well as in men. Nevertheless, the present sample is representative of an urban-dwelling population. Our data suggest that 8-oxodG levels are associated with systolic blood pressure and pulse pressure, indicators of vascular disease, whereas hsCRP is associated with metabolic factors. These data shed new light on the complex interplay between inflammation and oxidative stress in CVD.

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Disclosures

None.

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Arteriosclerosis, Thrombosis, and Vascular Biology



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Association of Oxidative DNA Damage and C-Reactive Protein in Women at Risk for Cardiovascular Disease

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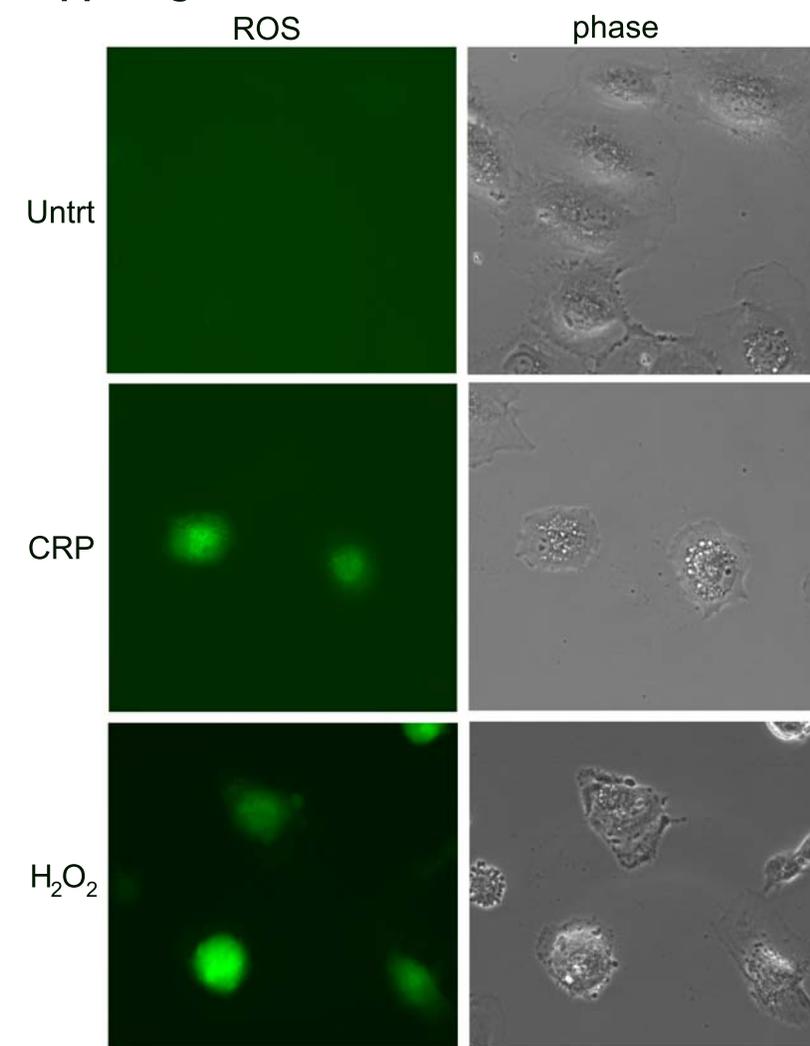
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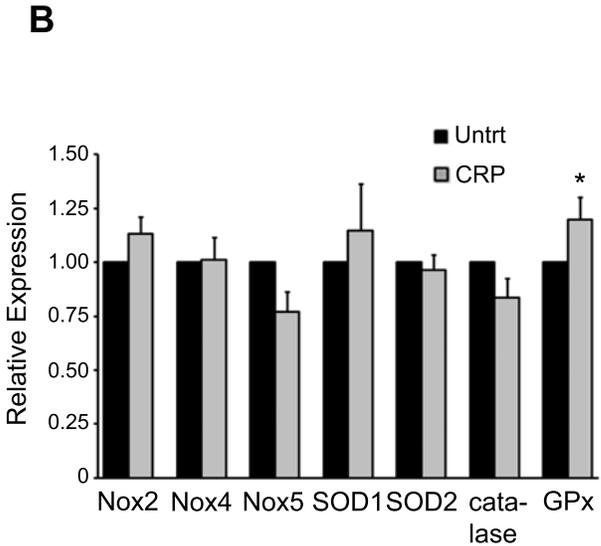
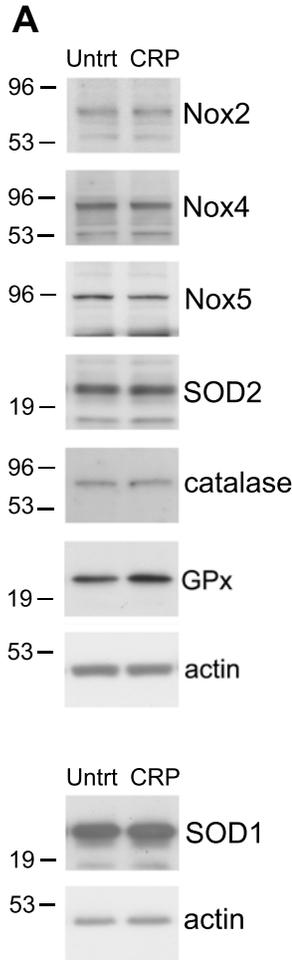
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Supplemental Material

Suppl. Figure S1

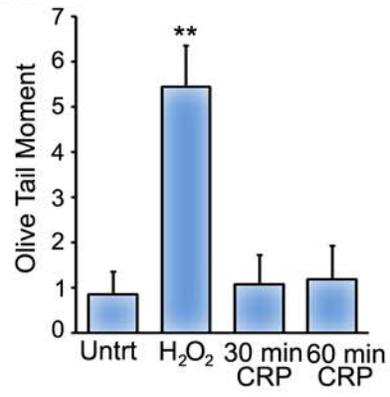


Suppl. Figure SII

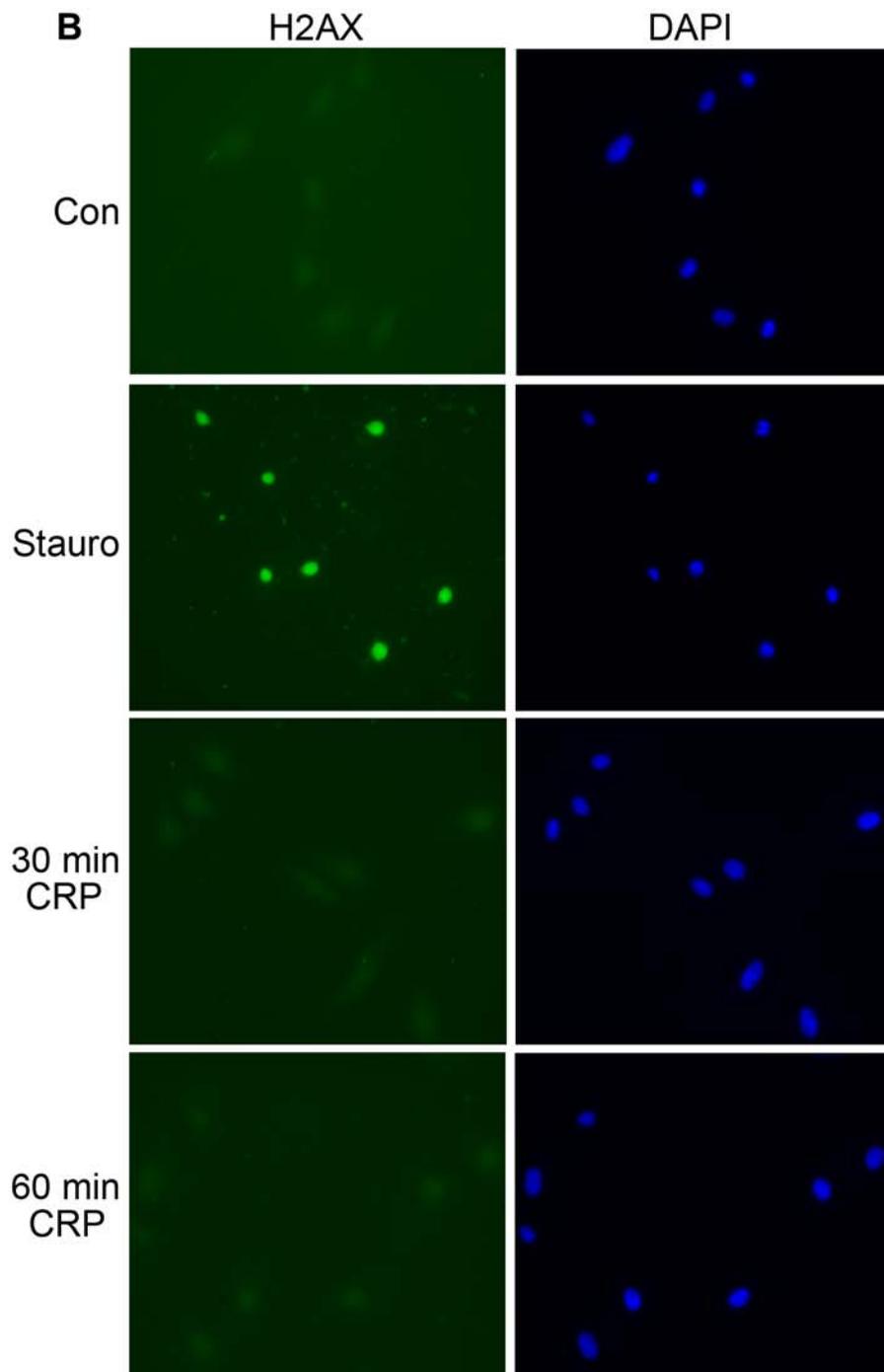


Suppl. Figure SIII

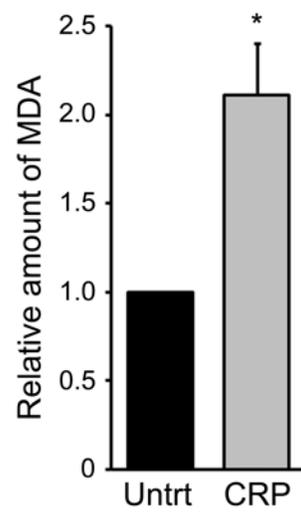
A



B



Suppl. Figure SIV



Supplementary Figure Legends

Supplemental Figure SI. hsCRP induces ROS in HUVECs. HUVECs were plated on chamber slides and 18 hrs later cells were incubated with the Oxidative Stress Detection Reagent from the Total ROS detection kit (Enzo Life Sciences) in serum-free media for 30 min. Cells were washed with PBS and were either mock treated (Untrt) or treated for 10 min with either 25 µg/ml hsCRP or 100 µM H₂O₂. Fluorescent images (visualizing ROS) or phase pictures were taken on a Zeiss Observer D1 microscope with an AxioCam1Cc1 camera.

Supplemental Figure SII. Effects of hsCRP on the expression of NADPH oxidases and antioxidant enzymes. (A) HUVECs were incubated in a 1:10 dilution of growth media for 18 hrs in the absence (Untrt) or presence of hsCRP (25 µg/ml). Cells were lysed, separated by SDS-PAGE and immunoblotted with anti-Nox2 (Abcam), anti-Nox4 (Santa Cruz), anti-Nox5 (Abcam), anti-catalase (Calbiochem), anti-SOD1 (Calbiochem), anti-SOD2 (Abcam), anti-glutathione peroxidase (GPx; Abcam), and anti-actin (Santa Cruz) antibodies as a protein loading control. To examine SOD1 expression, we loaded only 1/6 of the sample volume (lower immunoblots). (B) Relative levels of the indicated proteins were quantified from immunoblots and normalized to actin control. The histogram represents the normalized mean from 4 independent experiments + SEM. *P<0.05 by Student's t-test.

Supplemental Figure SIII. Effects of hsCRP on different forms of DNA damage. (A) HUVECs were mock treated or treated with 100 µM H₂O₂ or 25 µg/ml hsCRP for 30 or 60 min in serum-free media. The alkaline comet assay was performed as previously described^{1,2}. The comets were visualized using an Eclipse E-400 fluorescence microscope (Nikon, Japan) attached to a Pulnix video camera (Kinetic Imaging, LTD, Liverpool, UK), and were analyzed using Komet 5.5 software (Kinetic Imaging LTD). Olive tail moment was used as a measure of DNA damage level^{3,4}. Analogous results were obtained when % tail DNA was examined. The histogram represents the mean ± SEM from four experiments. **P<0.01 by one-way ANOVA and Tukey's post-hoc test. (B) HUVECs were treated with 25 µg/ml hsCRP for the indicated time points or with 2 µM staurosporine for 4 hrs. Cells were stained with anti-γ-H2AX antibodies (Millipore; Cat # 16-202A (clone JBW301)) or DAPI according to manufacturer's instructions.

Supplemental Figure SIV. hsCRP increases MDA levels in HUVECs. HUVECs (2 15 cm dishes/condition) were untreated or treated with 25 µg/ml hsCRP for 30 min in SF media. Cells were washed in PBS, sonicated, centrifuged at 3000 x g for 10 min and the supernatant was used directly for MDA analysis using the BIOXYTECH[®] MDA-586 kit according to manufacturer's instructions (OxisResearch[™]). MDA concentration was calculated according to a standard curve and was normalized to the amount of protein in the sample. The histogram represents the normalized mean + SEM from 4 independent experiments. *p<0.05 using Student's t-test.

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