The C-Reactive Protein + 1444C/T Alteration Modulates the Inflammation and Coagulation Response in Human Endotoxemia

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Background: C-reactive protein (CRP) plays a major role in the immune system and is an independent risk marker of cardiovascular disease. However, CRP’s role in atherogenesis as innocent bystander, causative, or even protective agent, remains unresolved. The +1444C/T alteration in the CRP gene has been reported to determine basal CRP concentrations. We hypothesized that this alteration may also be associated with the degree of inflammatory response and coagulation activation in a well-standardized model of systemic inflammation.

Methods: We administered 2 ng/kg endotoxin [Escherichia coli bacterial lipopolysaccharide (LPS)] intravenously to stimulate inflammation in 91 healthy young Caucasian male paid volunteers (age range, 19–40 years). Participants were confined to bed rest and fasted for 8.5 h after LPS infusion. We collected blood samples before LPS infusion and at 0, 2, 6, and 24 h after LPS infusion to measure inflammation markers [interleukin 6 (IL6), tumor necrosis factor-α (TNFα)], temperature, and coagulation markers (prothrombin fragment F1+2, D-dimer). We analyzed the CRP 3’ untranslated variant with a mutagenic separated PCR assay.

Results: Basal concentrations of high-sensitivity CRP were ~40% lower in +1444CC alteration carriers than in T homozygous (TT) allele carriers (P = 0.04). In contrast, basal IL6 concentrations were 2-fold higher in wild-type C homozygous (CC) than in TT individuals (P = 0.01). In response to the LPS challenge, CC individuals had 4-fold higher peak TNFα concentrations (P < 0.01), >2.5-fold higher peak IL6 concentrations (P < 0.01), and increased temperature (P < 0.01). Twenty-four hours after LPS challenge, prothrombin fragment F1+2 concentrations were 75% higher and D-dimer concentrations 50% higher in CC than in TT individuals (P < 0.05).

Conclusions: Genetic factors regulating CRP concentrations also modulate the individual response to endotoxin-stimulated inflammation.

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C-reactive protein (CRP) is a recognized acute-phase protein and a very sensitive marker of systemic inflammation and tissue damage (1). A member of the calcium-dependent ligand-binding pentraxin family, CRP binds with highest affinity to phosphocholine residues and is not merely a bystander molecule in inflammation, but an integral part of the innate immune system, actively contributing to host defense during infection. In addition, by binding to apoptotic cells, CRP promotes their opsonization and phagocytosis by macrophages. Because CRP binds to oxidized LDL, VLDL, and phosphatidyl choline as well as to damaged cell membranes, it is not surprising that CRP is found in atherosclerotic plaques (2) and in acute myocardial infarction lesions. Recently, increased serum CRP concentrations have been reported to be valuable for the prediction of atherothrombotic events and the outcome of acute coronary syndromes (3). Extensive studies (3, 4) of high-sensitivity CRP (hsCRP) have demonstrated that this inflammation marker independently predicts cardiovascular risk not detected by routine risk markers, adds prognostic information to risk...
assessment, and predicts long-term cardiovascular risk in individuals with no prior evidence of cardiovascular disease.

CRP serum concentrations are regulated primarily by proinflammatory cytokines that are released after an acute-phase reaction and that induce CRP de novo synthesis in the liver. Serum CRP concentrations begin to rise after ~6 h (5) and are widely used for diagnosis and monitoring of inflammatory diseases.

CRP baseline concentrations are influenced by various chronic microbial infections (6), a proatherogenic metabolic state (7), smoking (8), coffee consumption (9), stress (10), oral contraceptives (11), and menstrual cycle (12).

Baseline CRP serum concentrations seem to be heritable (13). Genetic variations in the interleukin (IL)-6*, IL-1, tumor necrosis factor alpha (TNF), and CRP genes may influence the induction of CRP synthesis (13, 14).

The human CRP gene is located on chromosome 1q21-q23, within a conserved genetic region that encodes for proteins critical to the immune system and to intercellular communication (6). CRP serum concentrations in acute and chronic inflammation show considerable interindividual variability, and these differences may be partly determined by genetic variation. Indeed, several alterations have been identified, and several small studies have reported that alterations within the CRP gene are associated with plasma CRP concentrations (13–15). A C/T alteration at position +1444 within the 3’ untranslated region of the CRP gene was recently reported to be associated with increased plasma CRP concentrations in 250 study participants (13).

Numerous immune modulatory effects of CRP have been described. We hypothesized that the CRP genotype might influence inflammatory cytokine expression and other markers of inflammatory response such as markers of coagulation activation. Experimental bacterial lipopolysaccharide (LPS) stimulation in healthy individuals has been proven as an excellent in vivo model to study the cytokine cascade underlying inflammation and has been applied in numerous studies (16–20). We evaluated the use of this standardized model to examine a putative association between the CRP +1444C/T alteration and inflammation or activation of coagulation in experimental LPS-induced endotoxemia.

**Materials and Methods**

**STUDY PARTICIPANTS**

The study protocol was approved by the Ethics Committee of the Medical University of Vienna, and all participants gave written informed consent to participate in the study. We obtained complete data and DNA samples from 91 healthy Caucasian male paid volunteers who had participated in already published and still unpublished clinical trials in which they had received LPS (21–24).

All participants were nonsmokers, 19–40 years of age, with a body mass index in the 15th to 85th percentile. Determination of health status included a medical history, physical examination, laboratory values, and virus and standard drug screening. Exclusion criteria were regular or recent intake of medications, including nonprescription medications, and clinically relevant abnormal findings in the medical history or laboratory values.

**STUDY PROTOCOL**

The experimental procedures of our LPS infusion studies have been described elsewhere (25). Briefly, volunteers were admitted to the study ward at 0800 h after an overnight fast. Participants were confined to bed rest throughout the entire study period and fasted for 8.5 h after LPS infusion. Participants received an intravenous bolus containing 2 ng/kg LPS (National Reference Endotoxin, Escherichia coli USP Convention Inc.).

**BLOOD SAMPLING AND ANALYSIS**

Blood was collected at times determined by the observed kinetics of plasma IL6 and TNFα in previous trials (25). Blood samples were collected by venipuncture into Becton Dickinson Vacutainer tubes containing EDTA anticoagulant before LPS infusion and thereafter at the times indicated in Fig. 1. Plasma samples were processed immediately by centrifugation at 2000g at 4 °C for 15 min and stored at −80 °C before analysis. Plasma IL6 and TNFα were analyzed with a high-sensitivity enzyme immunoassay (R&D-Systems). Plasma concentrations of F1,2 and D-dimer were evaluated by immunoassays (F1,2, Behring, Marburg; D-dimer, Boehringer Mannheim) as described previously (22).

To overcome the narrow measuring interval (0.16–10 ng/L), we analyzed the 2- and 6-h post-LPS samples after adequate dilution. CRP invariably increased 24 h after endotoxin administration and was an integral measure of IL6 bioactivity (26). Therefore, we measured hsCRP with an immunonephelometric test 24 h after LPS infusion (26).

**PCR ANALYSIS OF THE CRP 1444 ALTERATION**

DNA was isolated from EDTA blood by standard procedures. For detection of the +1444C/T variant in the 3’UTR of the CRP gene [rs1130864 in the National Center for Biotechnology Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/SNP)], the principle of mutagenic separated PCR was adapted as described (27, 28).

Mutagenic separated PCR is a single-tube PCR-based technique relying on allele-specific primers that differ in length by 9 bp, resulting in PCR products of different sizes. Single-base mismatches in the allele-specific prim-
ers introduce deliberate differences into the allelic PCR products to minimize crossreactions in subsequent cycles. One or 2 different PCR products are amplified, depending on the genotype. PCR products were generated in 25-μL volumes containing 0.65 units of AmpliTaq Gold (Perkin-Elmer Cetus), 1.5 mmol/L MgCl₂, 2 μmol/L of each dNTP (Amersham Pharmacia Biotech), 12 pmol CRP 1444 C primer (5’-CGT TAA CTA TGC TGG GAG AC-3’), 5 pmol CRP 1444 T primer (5’-CTG GGA GCT ATT TAA CTA TGC TGG GTA AT-3’), and 1 pmol common reverse primer (5’-CTT CTC AGC TCT TGC CTT ATG AGT-3’), all synthesized by TIB Molbiol, and 50 ng DNA. Amplifications were performed in an Eppendorf Thermo Cycler. A 10 min denaturation period at 95 °C was followed by 37 cycles of 95 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s. A final extension step of 7 min at 72 °C completed the reaction. For the CRP 1444 C allele, a 153-bp PCR product and for the CRP 1444 T allele, a 162-bp product were generated and separated on precast 10% Tris-borate-EDTA polyacrylamide gels (Bio-Rad Inc.) for 75 min at 175 V. After being stained with Sybr Green (Molecular Probes) for 20 min, bands were visualized on an ultraviolet transilluminator at 306 nm and photographed.

Data analysis
Data are expressed as the mean (SE). After repeated measures ANOVA, we applied nonparametric statistics for robustness. We used the Kruskal Wallis ANOVA for all statistical comparisons between groups, and the Mann–Whitney U-test for post hoc comparisons. All statistical calculations were performed with commercial statistical software (SPSS 10.0, SPSS Inc.).

**Results**
Of the 91 healthy volunteers who received LPS, 41 (45%) were wild-type homozygous +1444C genotype (CC), 37 (41%) were heterozygous +1444CT (CT), and 13 (14%) were homozygous +1444T (TT).

Genotype frequencies were in Hardy-Weinberg equilibrium in all groups and were similar to those reported previously (13).

**Table 1. CRP +1444 genotype-dependent inflammatory markers in healthy volunteers (n = 91).**

<table>
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<tr>
<th></th>
<th>Basal Concentrations</th>
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<tr>
<td></td>
<td>CRP +1444 CC (n = 41)</td>
<td>CRP +1444 CT (n = 37)</td>
<td>CRP +1444 TT (n = 13)</td>
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<tr>
<td>hsCRP, mg/L</td>
<td>2.8 (0.5)</td>
<td>3.7 (0.5)</td>
<td>4.6 (1)</td>
<td>0.04</td>
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<tr>
<td>IL-6, pg/mL</td>
<td>1.9 (0.3)</td>
<td>1.5 (0.4)</td>
<td>0.9 (0.2)</td>
<td>0.01</td>
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<tr>
<td>TNF, ng/L</td>
<td>2.8 (0.5)</td>
<td>3.0 (1.4)</td>
<td>2.3 (0.7)</td>
<td>0.5</td>
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<tr>
<td>D-Dimer, mg/L</td>
<td>0.22 (0.03)</td>
<td>0.16 (0.02)</td>
<td>0.17 (0.03)</td>
<td>0.2</td>
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<tr>
<td>F₁₋₂, nmol/L</td>
<td>0.59 (0.03)</td>
<td>0.55 (0.02)</td>
<td>0.60 (0.07)</td>
<td>0.5</td>
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<tr>
<td>Body temperature, °C</td>
<td>36.3 (0.1)</td>
<td>36.3 (0.1)</td>
<td>36.5 (0.0)</td>
<td>0.4</td>
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<tr>
<td>Monocyte counts, 10⁹/L</td>
<td>0.59 (0.05)</td>
<td>0.55 (0.03)</td>
<td>0.50 (0.05)</td>
<td>0.8</td>
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<thead>
<tr>
<th></th>
<th>Peak concentrations</th>
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<tr>
<td></td>
<td>CRP +1444 CC (n = 41)</td>
<td>CRP +1444 CT (n = 37)</td>
<td>CRP +1444 TT (n = 13)</td>
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<tr>
<td>hsCRP, mg/L</td>
<td>22.7 (2.1)</td>
<td>25.0 (2.7)</td>
<td>33.1 (6.9)</td>
<td>0.6</td>
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<td>24 h after LPS</td>
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<tr>
<td>IL-6, pg/mL</td>
<td>536 (101)</td>
<td>282 (69)</td>
<td>195 (123)</td>
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<tr>
<td>2 h after LPS</td>
<td>174 (32)</td>
<td>112 (28)</td>
<td>40 (12)</td>
<td>0.007</td>
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<tr>
<td>TNF, ng/L</td>
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<tr>
<td>2 h after LPS</td>
<td>1.2 (0.3)</td>
<td>0.9 (0.2)</td>
<td>0.8 (0.1)</td>
<td>0.7</td>
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<tr>
<td>D-Dimer, mg/L</td>
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<td>10 h after LPS</td>
<td>5.8 (1.4)</td>
<td>4.1 (0.8)</td>
<td>3.1 (0.4)</td>
<td>0.5</td>
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<tr>
<td>F₁₋₂, nmol/L</td>
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<tr>
<td>6 h after LPS</td>
<td>37.0 (0.1)</td>
<td>36.3 (0.1)</td>
<td>36.3 (0.2)</td>
<td>0.01</td>
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<tr>
<td>Body temperature, °C</td>
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<tr>
<td>6 h after LPS</td>
<td>Monocyte counts, 10⁹/L</td>
<td>1.01 (0.1)</td>
<td>0.87 (0.10)</td>
<td>0.7</td>
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A. Basal Concentrations
B. Peak concentrations

All concentrations are given as mean (SE), and P values are calculated by Kruskal-Wallis ANOVA.

a P≤0.05.
b P≤0.01.
CRP concentrations showed an upward tendency 24 h after LPS challenge [CC < CT < TT: 22.7 (2) mg/L in CC, 25.0 (3) mg/L in CT, and 33.1 (6) mg/L in TT individuals].

Association of CRP +1444 C/T alteration and inflammation markers

At baseline, before LPS infusion, IL6 basal concentrations in wild-type +1444 CC individuals were 2-fold higher than those in TT individuals (Table 1).

In response to LPS challenge, CC individuals had 2-fold higher peak IL6 concentrations (P < 0.01) and higher IL6 release at all time points (P < 0.05) than TT carriers (Fig. 1). Similarly, peak TNFα concentrations were 4-fold higher (P < 0.01), and body temperature (P < 0.01) was higher in CC than in TT individuals (Fig. 1). Interestingly, basal IL6 concentrations correlated with both peak TNFα concentrations (r = 0.25, P = 0.03) and IL6 concentrations (r = 0.37, P = 0.001), whereas basal TNFα concentrations did not correlate with peak concentrations. Monocyte counts, however, did not differ between groups (Table 1).

Markers of coagulation activation — association of the +1444 C/T CRP alteration

Plasma F1+2 concentrations increased 8-fold 6 h after LPS infusion (P < 0.05 vs baseline). Similarly, D-dimer concentrations increased 5-fold, with a maximum at 10 h after LPS infusion (P < 0.05 vs baseline). Although peak concentrations did not differ significantly between genotypes, significant differences were seen 24 h after LPS infusion; plasma concentrations of prothrombin fragment F1+2 were higher in CC individuals [1.0 (0.1) nmol/L] than in CT individuals [0.7 (0.3) nmol/L at 24 h, P < 0.05] and TT carriers [0.6 (0.1) nmol/L at 24 h, P < 0.05, Fig. 1]. In parallel, 24 h after LPS infusion, D-dimer concentrations were higher in CC individuals [0.74 (0.1) mg/L] than in TT individuals [0.52 (0.08) mg/L, P < 0.05 between groups].

Genotype strata differed particularly in their cytokine response, and to a lesser degree in their coagulation response, to LPS infusion. For all measured variables we observed a consistent gene dosage effect between groups (Fig. 1).

Discussion

Baseline serum CRP concentrations predict the risk of future myocardial infarction and stroke and reflect a baseline inflammation level (4, 29). Recent studies indicate that, independent of inflammation, genetic variations in the CRP gene, as well as in the IL-6 and IL-1 genes, influence circulating CRP concentrations (14, 30, 31).

Several CRP variants have been associated with interindividual differences in CRP serum concentrations (14, 32, 33). In the healthy young men of our study, baseline CRP concentrations were 64% higher for TT than CC genotype individuals, in agreement with a previous report (14). Similarly, CRP concentrations after LPS challenge were somewhat higher in TT than in CC individuals.

The major novel finding of this study is a hitherto undiscovered association of the CRP +1444 alteration...
with inflammation, and to a lesser degree, with coagulation. In contrast to CRP, the cytokine release to LPS was severalfold higher in healthy CC individuals than in TT individuals. In CC individuals, released amounts of TNFα, IL-6, and markers of coagulation activation were significantly higher than in TT individuals (Fig. 1, Table 2). Heterozygous individuals exhibited an intermediate response for all variables, supporting the concept of a consistent gene dosage effect. Thus, the +1444 CRP TT genotype associated with the highest basal CRP concentrations was also associated with the lowest measured release of cytokines and coagulation markers, and vice versa. This is noteworthy because CRP release is primarily IL-6–dependent in this model of human endotoxemia (21). The decreased IL-6 release in TT individuals could reflect their lower baseline IL-6 concentrations. It may also reflect lower TNFα release, because IL-6 concentrations are dependent on TNF action in the LPS infusion model (34, 35). In contrast, the coagulation response is not dependent on TNF or IL-6 in human endotoxemia (21, 35, 36). Therefore, it is possible that a more upstream pathway common to both biological systems is affected.

Considerable recent debate has revolved around whether CRP by itself is causally involved in inflammatory processes and atherogenesis (37), is an innocent bystander (38), or exerts protective antiinflammatory effects (39–41). In particular, the beneficial effects—Mainz hypothesis—have recently been discussed extensively in the literature (40, 42).

Our results indicate that gene alterations leading to increased CRP expression might have antiinflammatory effects, at least after LPS stimulation. Our observations may also explain why functional genetic alterations within the CRP gene, which lead to increased CRP (e.g., the 1059G/C variant), are not major determinants for cardiovascular risk (43). This paradox may be attributable to the antiinflammatory effects of alteration(s) associated with mildly increased CRP concentrations. It will be interesting to explore whether mildly increased CRP concentrations have antiinflammatory actions. Such studies may become feasible once antagonists of CRP become available for use in clinical trials. Together with previous data (14, 43), our results support the concept that genotype-dependent reference intervals for CRP might increase the predictive power and precision of cardiovascular risk prediction.

LIMITATIONS OF THE STUDY

In our trial we studied genotype-dependent CRP expression in healthy young Caucasian males. Our study population included a relatively homogeneous group of healthy, young, nonsmoking, nonobese men. Thus, it was not possible to investigate the modulation of the inflammation response by other variables, including age, menopausal state, ethnic background, body mass index, or smoking, that have been reported to influence the genotype-dependent individual expression of CRP (44). Another limitation of our study was the small sample size and the restriction to 1 inflammatory stimulus, LPS, administered as a single dose. We cannot exclude different results generated in response to other complex multifactorial proinflammatory stimuli over a long time-span that may be present in inflammatory diseases such as arteriosclerosis or diabetes. Finally, we cannot determine whether the examined alteration or another alteration in linkage disequilibrium is causing the marked differences in inflammatory response to LPS challenge.

In conclusion, genetically-determined regulators of CRP concentrations also modulate the individual response to a key inflammatory stimulus, endotoxin. This genetic determination could explain the discrepant results indicating that carriers of CRP gene variants clearly associated with increased basal CRP concentrations such as +1444 CT are not necessarily at an increased risk for cardiovascular disease (43, 45).

References


