Persistent Activation of Nuclear Factor Kappa-B Signaling Pathway in Patients With Unstable Angina and Elevated Levels of C-Reactive Protein

Evidence for a Direct Proinflammatory Effect of Azide and Lipopolysaccharide-Free C-Reactive Protein on Human Monocytes Via Nuclear Factor Kappa-B Activation

Giovanna Liuzzo, MD, PhD,* Matteo Santamaria, MD,* Luigi M. Biasucci, MD, FACC,* Michela Narducci, PhD,* Valeria Colafrancesco, PhD,* Annalisa Porto, PhD,*† Salvatore Brugaletta, MD,* Michela Pinnelli, MD,* Vittoria Rizzello, MD,* Attilio Maseri, MD, FACC,† Filippo Crea, MD, FACC*

Rome and Milan, Italy

Objectives Our study investigated: 1) the contribution of nuclear factor kappa-B (NF-κB) signaling pathway to the enhanced inflammatory response observed in unstable angina (UA) patients with elevated levels of C-reactive protein (CRP); and 2) whether CRP may have direct proinflammatory effects via NF-κB activation.

Background Unstable angina patients with elevated CRP have enhanced inflammatory response and increased risk of persistent instability, myocardial infarction, and death.

Methods We studied 28 patients with history of UA and persistently elevated CRP (>3 mg/l) followed for 24 months and free of symptoms for at least 6 months (group 1), 14 patients with history of UA and low CRP (group 2), and 24 patients with chronic stable angina and low CRP (group 3). Peripheral blood monocytes were analyzed for spontaneous NF-κB activation and interleukin (IL)-6 and tumor necrosis factor (TNF)-α production. To assess the direct proinflammatory effects of CRP, monocytes from 8 healthy subjects were stimulated in vitro with increasing doses of CRP (5 to 10 to 25 μg/ml), lipopolysaccharide (LPS) (1 to 10 ng/ml), or both.

Results Spontaneous NF-κB activation in vivo was demonstrated in 82% of group 1 versus 14% of group 2 and 21% of group 3 patients (p < 0.001). Interleukin-6 and TNF-α production was significantly correlated with the NF-κB activation status (r = 0.55, p < 0.001 and r = 0.53, p = 0.006, respectively). Patients with NF-κB activation had recurrence of acute coronary events (60% vs. 28%; p = 0.017). C-reactive protein induced a significant but modest in vitro NF-κB activation in human monocytes (p = 0.002). Coincubation with LPS produced a greater-than-additive response (p < 0.01 vs. CRP and LPS alone).

Conclusions Nuclear factor kappa-B activation might represent a mechanism by which CRP amplifies and perpetuates the inflammatory component of acute coronary syndromes and influences the clinical outcome. (J Am Coll Cardiol 2007;49:185–94) © 2007 by the American College of Cardiology Foundation

Growing evidence suggests that C-reactive protein (CRP) predicts short- and long-term outcome in patients with acute coronary syndromes (ACS), independently of other biohumoral risk markers (1–6). Furthermore, patients with unstable angina (UA) and persistently high levels of CRP exhibit enhanced inflammatory cell reactivity both in vivo in response to coronary angiography, percutaneous coronary intervention (7) and myocardial necrosis (8) and in vitro in response to lipopolysaccharide (LPS) challenge (9).
Patients were followed for a further period of 12 months after enrollment to assess the clinical outcome. Follow-up visits, consisting of physical examination, a standard 12-lead electrocardiogram, and a treadmill stress test, were performed every 3 months. Recurrence of new acute coronary events (cardiac death, myocardial infarction, and coronary care unit admission for UA) was recorded.

PROTOCOL B. To assess whether CRP may have direct proinflammatory effects on human circulating mononuclear cells via NF-κB activation, a smaller study was designed including 8 healthy volunteers (mean age 49 ± 5 years, 7 men) without coronary risk factors. In particular, subjects with acute or chronic inflammatory or infectious diseases, malignancies, immunologic or hematologic disorders, treatment with anti-inflammatory drugs, or recent (<6 months) surgery or trauma were excluded.

All subjects gave their written informed consent. The ethics committee of the Catholic University of Rome approved the study.

Blood sampling and monocyte isolation. PROTOCOL A. In all patients, a venous blood sample was obtained at the time of study entry for the following analyses: 1) CRP plasma levels and IL-6 and TNF-α serum levels; 2) total and differential white blood cell counts; and 3) monocyte isolation. In 16 patients of group 1, 10 patients of group 2, and 14 patients of group 3, a further blood sample for total and differential white blood cell counts and monocyte isolation was obtained at 12 months follow-up.

Isolation of peripheral blood monocytes was performed by gradient centrifugation layering 30 ml heparinized (10 U/ml) blood over NycoPrep (Life Technologies, Rockville, Maryland) (9). Isolation of ≥80% pure monocytes was obtained, as evaluated by using flow cytometry (data not shown).

Freshly isolated peripheral blood monocytes (5 × 10⁵) were analyzed for spontaneous NF-κB activation (see succeeding text) and for IL-6 and TNF-α production after in vitro stimulation, as previously described (9). Briefly, monocytes were incubated for 4 h at 37°C in RPMI-1640 supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.2 mmol/l L-glutamine. Cells were stimulated with low dose of LPS (1 mg/ml, Escherichia coli 011:B4; Sigma Chemical Co., St. Louis, Missouri), which reflects the LPS concentration detected during clinical infections (23). After 4 h incubation, the culture supernatant was removed and stored at −80°C for further analysis.

PROTOCOL B. In the 8 healthy volunteers, 40 ml heparinized venous blood was obtained at the time of study entry for monocyte isolation, which was performed as described in protocol A. In vitro studies were carried out using recombinant human C-reactive protein (rhCRP) and/or highly purified CRP isolated from human serum, as specifically indicated. Recombinant human CRP (from Escherichia coli, >99% of purity) was obtained from Calbiochem (La Jolla, California) and was supplied in 20 mmol/l Tris-HCl, pH 7.5, 140 mmol/l NaCl, 2 mmol/l CaCl₂, and 0.05% NaN₃. Recombinant

Methods

Population. PROTOCOL A. Three groups of subjects were prospectively studied. Group 1 included 28 consecutive patients (mean age 64 ± 10 years, 17 men) with a history of UA and persistently elevated levels of CRP (>3 mg/l) over the following 24 months, measured in plasma samples obtained at the time of hospital admission, at discharge, and every 3 months after discharge. Group 2 included 14 consecutive patients (mean age 66 ± 9 years, 10 men) with a history of UA and persistently normal levels of CRP over the following 24 months, measured at the same time points as in group 1. Group 3 included 24 consecutive patients (mean age 59 ± 9 years, 19 men) with stable angina and normal CRP (<3 mg/l).

All patients fulfilled the following inclusion criteria: 1) no acute coronary events in the 6 months before study entry; 2) no evidence of inflammatory or infectious diseases, malignancies, or immunologic or hematologic disorders; 3) no treatment with anti-inflammatory drugs other than low-dose aspirin; 4) ejection fraction >40%; and 5) age <75 years.

Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS</td>
<td>acute coronary syndromes</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-B</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>UA</td>
<td>unstable angina</td>
</tr>
</tbody>
</table>

Promoting the expression of a cascade of procoagulant and proinflammatory genes in response to a variety of stimuli (10–13). Accordingly, Ritchie (14) has demonstrated that NF-κB is activated in circulating leukocytes of patients with ACS but not in leukocytes of stable angina patients, and Cominacini et al. (15) have recently found that the activation of NF-κB in monocytes of UA is, at least in part, induced by circulating molecules such as oxidized low-density lipoproteins (ox-LDL). Activated NF-κB has been identified in monocyte/macrophages, smooth muscle cells, and endothelial cells in human atherosclerotic vessels, but not in healthy vessels (11), and is enhanced in coronary plaques of patients with UA (16). Coronary plaques from patients with ACS exhibit increased expression of several NF-κB–regulated genes, including proinflammatory cytokines, growth factors, chemokines, adhesion molecules, and tissue factor (12). C-reactive protein is also present in atherosclerotic plaques (17–19) and exhibits a range of proinflammatory properties that could directly contribute to pathogenesis, progression, and complications of the atheroma (20–22).

Therefore, the aim of the present study was 2-fold: 1) to explore the contribution of NF-κB signaling pathway to the enhanced inflammatory response observed in patients with UA and elevated levels of CRP (protocol A); and 2) to assess whether CRP may have direct proinflammatory effects on human circulating leukocytes via NF-κB activation (protocol B).
human CRP was used in electrophoresis mobility shift assay (EMSAs). Highly purified (>99%) native pentameric CRP isolated from human serum was also obtained from Calbiochem and was supplied in 10 mmol/l Tris, pH 8.0, 140 mmol/l NaCl, 2 mmol/l CaCl₂, and 0.1% NaN₃. Purified CRP was used for enzyme-linked immunosorbent assay (ELISA) and confocal microscopy.

Aliquots of 5 × 10⁵ cells were incubated at 37°C in phosphate-buffered solution (PBS) and stimulated with increasing doses of CRP alone (5 to 10 to 25 µg/ml), either rhCRP (EMSA) or purified CRP (ELISA, confocal microscopy) (see succeeding text), LPS alone (10 ng/ml), or both for 30 min to assess the effects on NF-κB activation. A cell aliquot was preincubated with 50 mmol/l pyrrolidine dithiocarbamate (PDTC) (Calbiochem, La Jolla, California), a NF-κB inhibitor, for 1 h before CRP stimulation (25 µg/ml) for 30 min. Cells were also stimulated with CRP (25 µg/ml) denatured by incubation in boiling water bath for 1 h.

**Specificity of the CRP preparations used for in vitro studies.** The following strategies were used to determine the specificity of both the CRP preparations used for in vitro studies: 1) the purity of CRP preparations was further confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (a single band on silver-stained and overloaded gels); 2) to remove the azide from CRP preparations, 1 mg was dialyzed twice against 500 ml of the Tris/NaCl/CaCl₂ buffer; 3) endotoxin was removed from CRP with a detoxigel column (Pierce Biochemicals, Rockford, Illinois), and all the reagents used were endotoxin free according to the limulus test (sensitivity 0.06 U/ml; Sigma); 4) the cell cultures stimulated with CRP were coincubated with Polymixin B (10 µg/ml) to insure that CRP-induced effects were distinct from those mediated by LPS; and 5) CRP was denatured by incubation in boiling water bath for 1 h (denatured CRP). The CRP doses used in this study corresponded to CRP plasma concentrations detected in UA patients (1–5).

**Measurements.** **ASSESSMENT OF NF-κB ACTIVATION.** Electrophoresis mobility shift assay (EMSAS). In unstimulated cells, NF-κB dimers are found in the cytoplasm in an inactive form bound to inhibitory proteins that prevent NF-κB dimers from entering the nucleus. Proinflammatory stimuli initiate a rapid cellular signaling pathway in which specific kinases phosphorylate the NF-κB inhibitory proteins, causing their rapid degradation. The release of NF-κB from the inhibitory proteins results in the passage of NF-κB dimers into the nucleus, where they bind to specific sequences in the promoter region of target genes, thus starting the process leading to protein synthesis. The activated form of NF-κB is a heterodimer that usually consists of a p65 and a p50 subunit (10).

Nuclear presence of NF-κB complexes can be demonstrated in mobility shift assays (24). Nuclear extracts from 5 × 10⁵ monocytes were prepared by use of a high-salt extraction protocol, and electrophoretic mobility shift assays were performed as described (25). Nuclear extract (5 µg) was combined with 15 µl binding buffer, 1.5 µg poly(dI-dC) (Sigma), and 1.5 µg nonspecific oligonucleotide (5’-TCGAAGTAC-TCATTGCTCGAGATCGAT-AGATCTGAA-TTCAGTACTCC-3’). In supershift assays, 1 µg of a p50 subunit-specific antibody (Upstate Biotechnology, Charlottesville, Virginia) or irrelevant mouse IgG1 (Sigma) was added to the reaction. Total volume of reaction mixture was adjusted to 25 µl, and the mixture was left on ice for 30 min. Specific double-stranded oligonucleotide probe corresponding to NF-κB binding site (5’-AGTTGAGGGACTTTTCCCAGGC-3’) (24) was radiolabeled with [γ-³²P] adenosine triphosphate (NEN, Boston, Massachusetts) by standard end-labeling reaction. Annealed probes at a final concentration of 40 fmol/µl were added to the reaction and incubated at room temperature for 30 min. Protein-DNA complexes were resolved on 6% nondenaturing polyacrylamide gels, visualized using a PhosphorImager screen (Molecular Dynamics, San Jose, California), and quantified using Imagequant software (National Institutes of Health, Bethesda, Maryland). As a positive control, we used nuclear extracts from 5 × 10⁵ monocytes incubated for 30 min with 10 ng/ml LPS and as a negative control nuclear extracts from 5 × 10⁵ monocytes incubated for 30 min with PBS only.

**ELISA for Active NF-κB p65.** The amount of activated NF-κB p65 subunit in nuclear extract (5 µg), prepared from 5 × 10⁵ monocytes (as described in preceding text) was assessed by a sensitive ELISA assay for active NF-κB (TRANS-AM, Active Motif, Rixensart, Belgium). The NF-κB ELISA kits contain a 96-well plate on which has been immobilized oligonucleotide containing the NF-κB consensus site (5’-GGGACTTTCC-3’). The active form of NF-κB contained in nuclear-cell extract specifically binds to this oligonucleotide. The primary antibodies used to detect NF-κB recognize an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. A horseradish peroxidase-conjugated secondary antibody provides a sensitive colorimetric readout that is easily quantified by spectrophotometry by reading within 5 min the absorbance at 450 nm with a reference wavelength of 650 nm. The assay is specific for NF-κB activation and has been shown to be 10-fold more sensitive and 40-fold faster than the gel retardation technique: detection limit <0.5 µg nuclear-cell extract/well; range of detection 0.2 to 5 µg of nuclear-cell extract/well. The Jurkat nuclear extract is used as a positive control for NF-κB activation. The wild-type consensus oligonucleotide is used as a competitor for NF-κB binding to monitor the specificity of the assay; at 20 pmol/well it will prevent NF-κB binding to the probe immobilized on the plate, and, conversely, the mutated consensus oligonucleotide has no effect on NF-κB binding.

**CONFOCAL MICROSCOPY.** The activation of NF-κB was also determined by confocal microscopy assessing the nuclear localization of the p65 NF-κB subunit. After stimulation with CRP alone (25 µg/ml), LPS alone (10 ng/ml), or both for 30 min, as described in the preceding text, aliquots of 5 × 10⁴ monocytes were resuspended in 200 µl RPMI 1640 with 1%
fetal calf serum and permitted to adhere to chamber slides (Nalgene Nunc) for 30 min at room temperature. Then cells were fixed in PBS + 4% formaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100, and stained. Cells were incubated for 1 h at room temperature with a 1:100 dilution of mouse monoclonal antihuman primary antibody for p65 (sc-8008; Santa Cruz Biotechnology, Santa Cruz, California) in blocking buffer, then they were rinsed and incubated in the dark for 1 h with a 1:100 dilution of FITC-conjugated goat antimouse IgG secondary antibodies. Nuclei were stained with the fluorescent dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The stained cells were analyzed with the Olympus FV500 confocal laser-scanning microscope (Olympus Deutschland, Hamburg, Germany); 3 images were obtained from different regions of each stained slide. Cells were scored as either positive or negative for nuclear p65, and the percentage of cells positive for nuclear p65 was determined. The percentage of cells with p65 subunit accumulated in the nucleus was calculated from at least 100 DAPI nuclei counted for each region.

MEASUREMENTS OF CRP AND CYTOKINES. CRP was measured with a high-sensitivity latex-enhanced immunonephelometric assays (Dade Behring, Marburg, Germany) (3). The median normal value for CRP is 0.8 mg/l, with 90% of all values in healthy subjects <3 mg/l. Interleukin-6 and TNF-α were measured with a commercial assay kit (Quantikine human IL-6 and Quantikine human TNF-α; R&D Systems, Minneapolis, Minnesota) (26). Interleukin-6 and TNF-α measurements were performed in duplicate, and the intra- and interassay variability was <10%. The range of values detected by the assay was 3 to 300 pg/ml for IL-6 and 15.6 to 1,000 pg/ml for TNF-α. Total and differential white blood cell counts were obtained with a hematology analyzer by using automated cytometry in flow.

Statistical analysis. Because CRP, IL-6, and TNF-α values do not follow a normal distribution, nonparametric tests were used (the Mann-Whitney U test for comparisons between groups and the Spearman rank correlation test to determine correlations). The remaining continuous variables were compared by using analysis of variance (ANOVA) for comparisons between different groups and ANOVA for repeated measures within groups, with the Tukey test correction as appropriate and as suggested by the statistical software used. Proportions were compared with the Fisher exact test. The CRP, IL-6, and TNF-α values are expressed as medians and ranges; the remaining variables are expressed as mean ± SD. A p value of <0.05 (2-tailed) was considered to be statistically significant. All statistical analysis was performed with SigmaStat software (SPSS Inc., Chicago, Illinois).

Results

Protocol A. Patient demographic and clinical data are summarized in Table 1.

Spontaneous NF-κB activation as assessed by EMSA was observed in 23 of 28 (82%) group 1 patients, in 2 of 14 (14%) group 2 patients, and in 5 of 24 (21%) group 3 patients (p < 0.001; group 1 vs. group 2 and group 3) (Fig. 1A). Quantification of activated NF-κB p65 subunit by ELISA showed a statistically significant difference between group 1 and the other groups (p = 0.009; ANOVA with Tukey test adjustment for comparisons between the 3 groups) (Fig. 1B). Moreover, after 12 months of follow-up NF-κB activation was detected in 81% of group 1 patients, 20% of group 2 patients, and 21% of group 3 patients who had a second determination of NF-κB activation status (p = 0.002; group 1 vs. group 2 and group 3). The concordance between the 2 determinations was almost complete (with only 1 patient of group 3 initially negative becoming positive).

In the overall population, IL-6 and TNF-α production in response to LPS (1 ng/ml) was significantly correlated with baseline levels of CRP (r = 0.49; p = 0.005; and r = 0.58; p = 0.003; respectively) and with the NF-κB activation status (r = 0.55; p < 0.001; and r = 0.53; p = 0.006; respectively). Accordingly, serum levels of CRP and production of IL-6 and TNF-α in response to LPS were significantly higher in patients with than in those without NF-κB activation (Fig. 2). The CRP levels were 10.4 (0.9 to 67.5) mg/l in patients with and 1.3 (0.2 to 10.8) mg/l in patients without NF-κB activation (p < 0.001). The IL-6 production was 7.5 (2.0 to 25.7) ng/ml in patients with and 2.4 (0.2 to 13.0) ng/ml in patients without NF-κB activation (p < 0.001). The TNF-α production was 1.4 (1.1 to 3.2) ng/ml in patients with and 1.1 (0.9 to 1.6) ng/ml in patients without NF-κB activation (p = 0.006). No differences were observed in total and differential white blood cell counts between patients with and patients without NF-κB activation (data not shown).

Finally, 18 of 30 patients (60%) with persistent activation of NF-κB versus 10 of 36 patients (28%) without NF-κB activation (p = 0.017) had recurrence of acute coronary events (14 patients with and 8 patients without NF-κB activation had recurrence of UA; 3 patients with and 2 patients without NF-κB activation had myocardial infarction; 1 patient with NF-κB activation died) during the follow-up period. None of the other analyzed clinical variables (demographic data, risk factors, medications [including statins], severity of coronary disease, previous PCI or CABG) was associated with the presence of NF-κB activation (data not shown).

Protocol B. Incubation of the radioactive NF-κB binding site-specific probe with nuclear proteins isolated from human monocytes resulted, when analyzed by EMSA, in a single shifted band that was barely detectable in unstimulated cells, was markedly induced by stimulation with LPS (10 ng/ml) as expected, but was also induced by stimulation with increasing doses of rhCRP (5 to 10 to 25 μg/ml), and inhibited by preincubation with PDTC (50 mmol/l) and by the use of denatured rhCRP (Fig. 3A). Coincubation of both rhCRP and LPS had a greater-than-additive effect (Fig. 3B). Gel supershift experiments with monoclonal
antibodies to p50 subunit indicated that complexes consisted of heterodimers containing the p50 subunit (Fig. 3). Quantification of the nuclear expression of the activated NF-κB p65 subunit was performed by ELISA and expressed as optical density (OD) at 450 nm. Purified CRP, used for the ELISA at the increasing doses of 5 to 25 μg/ml, induced a slight but significant increase in NF-κB p65 expression (p = 0.002; ANOVA for repeated measures with the Tukey test adjustment for the 10 comparisons included) up to a mean value of 0.257 ± 0.162 OD (159% over the basal value), which was significantly greater than that observed with LPS alone (p = 0.001) or CRP alone (p = 0.003) (Fig. 4B).

Moreover, in 3 healthy volunteers we analyzed the nuclear accumulation of p65 in the presence of purified CRP (25 μg/ml), LPS (10 ng/ml), or both, by using confocal microscopy of monocytes stained with FITC-labeled antibody against p65. To identify the nucleus, monocytes were stained with DAPI. In nonstimulated monocytes, p65 was distributed throughout the cytosol. After stimulation with purified CRP and LPS, p65 consistently disappeared from the cytosol and accumulated in the nucleus, especially after costimulation with both of them. After treatment of monocytes with denatured CRP, the pattern of the p65 subcellular distribution was similar to that in the nonstimulated cells (Fig. 5A). Then, cells were scored as either positive or negative for nuclear p65. The percentage of cells positive for nuclear p65 was 12% at baseline (untreated cells), 22% after CRP stimulation, 30% after LPS stimulation, and close to 50% after treatment with both CRP and LPS, and it was reduced to 16% when denatured CRP was used (p < 0.001; ANOVA for repeated measures with the Tukey test adjustment for the 10 comparisons included) (Fig. 5B).
Discussion

Our study demonstrates that, 6 months or longer after the last acute coronary event, in an apparently stable phase of the disease, circulating monocytes from UA patients with persistently elevated levels of CRP express in vivo activation and nuclear translocation of the transcription factor NF-κB. Moreover, CRP concentrations known to predict adverse clinical outcome in UA induce NF-κB activation in vitro, as consistently shown using 3 different methods. Although CRP-induced NF-κB activation is modest, our findings consistently show that

---

**Figure 1** Assessment of Spontaneous NF-κB Activation in Circulating Monocytes (Protocol A)

(A) Electrophoresis mobility shift assay (EMSA). In each patient, freshly isolated peripheral blood monocytes (5 × 10^5) were analyzed for the spontaneous presence of activated nuclear factor (NF)κB complexes in their nuclei by EMSA in the presence of anti-p50 subunit monoclonal antibodies or isotype-matched control IgG antibodies. As a positive control (Pos) nuclear extracts were generated from 5 × 10^5 monocytes incubated for 30 min with 10 ng/ml lipopolysaccharide (LPS), and as a negative control (Neg) nuclear extracts were generated from 5 × 10^5 monocytes incubated for 30 min with phosphate-buffered solution only. NFκB heterodimers containing the p50 subunit were detected in 82% of group 1 patients, 14% of group 2 patients, and 21% of group 3 patients (p < 0.001; group 1 vs. group 2 and group 3). The figure shows the assessment of NFκB activation in 3 patients (Pt) of group 1 and in 3 patients of group 2. (B) Enzyme-linked immunosorbent assay (ELISA) for active NFκB p65 subunit. The amount of activated NFκB p65 subunit in nuclear extract (5 μg), prepared from 5 × 10^5 monocytes was assessed by a sensitive ELISA assay for active NFκB. The activated NFκB p65 subunit was significantly higher in group 1 than in the other groups. ANOVA = analysis of variance; OD = optical density.

---

**Figure 2** Inflammatory Response According to the NF-κB Activation Status (Protocol A)

Serum levels of CRP (A), and production of IL-6 (B) and TNF-α (C) in response to in vitro LPS challenge were significantly higher in patients with (NFκB+) than in those without (NFκB−) NFκB activation. (B and C) Monocytes (5 × 10^5) were stimulated with a low dose of LPS (1 ng/ml); after 4 h of incubation, the culture supernatant was removed and stored at −80°C for IL-6 and TNF-α measurements by ELISA. CRP = C-reactive protein; IL = interleukin; TNF = tumor necrosis factor; other abbreviations as in Figure 1.
CRP is able to amplify the effect of a standard proinflammatory stimulus, such as a low dose of LPS, producing a greater-than-additive response. Thus, NF-κB activation might represent a mechanism by which CRP amplifies and perpetuates the inflammatory component of ACS, and influences the clinical outcome, because patients with NF-κB activation had recurrence of acute coronary events over 1 year of follow-up.

In 8 healthy volunteers, aliquots of $5 \times 10^5$ cells were incubated at 37°C in PBS and stimulated with increasing doses of recombinant human (rh) CRP (5 to 10 to 25 μg/ml) for 30 min to assess the effects on NF-κB activation. A cell aliquot was incubated with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (50 mmol/l) for 1 h before CRP stimulation (25 μg/ml), and another cell aliquot was stimulated with CRP (25 μg/ml) denatured by incubation in boiling water bath for 1 h (25den). As a positive control (Pos) nuclear extracts were generated from $5 \times 10^5$ monocytes incubated for 30 min with 10 ng/ml LPS, and as a negative control (Neg) nuclear extracts were generated from $5 \times 10^5$ monocytes incubated for 30 min with PBS only. As shown in this representative EMSA, NF-κB activation in circulating monocytes was induced by stimulation with increasing doses of rhCRP and inhibited by preincubation with PDTC (50 mmol/l) and by the use of denatured rhCRP (A); coincubation of both rhCRP and LPS had a greater-than-additive effect (B). Gel supershift experiments with monoclonal antibodies to the p50 subunit indicated that complexes consisted of heterodimers containing the p50 subunit. Other abbreviations as in Figures 1 and 2.
NF-κB is a pivotal transcription factor in plaque destabilization. NF-κB is a pivotal transcription factor involved in the induction of specific proinflammatory genes (10). It was recently suggested that NF-κB may be critical in plaque instability by transducing pathogenic stimulation to expression of genes that promote recruitment and activation of inflammatory cells in the coronary plaques (12). Activation of NF-κB may occur after stimulation of lesional cells by pathogenically relevant factors such as proinflammatory cytokines, TNF-α and IL-1β, bacterial LPS, viruses, components of microorganisms such as Chlamydia pneumoniae, and reactive oxygen species involved in low-density lipoprotein oxidation (11–13). Activated NF-κB has been identified in monocyte/macrophages, smooth muscle cells, and endothelial cells in human atherosclerotic vessels, but not in healthy vessels (11), and is enhanced in ACS (16). Plaques from patients with ACS exhibit increased expression of several NF-κB–regulated genes, including the cytokines TNF-α and IL-1α (which induce NF-κB activation in a positive feedback loop), IL-6 (the major inducer of acute-phase protein production), interferon-γ (the most powerful activator of mononuclear cells), growth factors (stimulating smooth muscle cell proliferation), chemokines, adhesion molecules, and tissue factor (a major initiator of the coagulation cascade) (12).

Our data confirm and expand the previous observation by Ritchie (14), who demonstrated NF-κB activation in circulating monocytes of patients with UA in the acute phase of instability. Indeed, we found NF-κB activation in a subset of patients with persistent elevation of CRP plasma levels long after an episode of coronary instability. We also found an association between persistent NF-κB activation and recurrence of acute coronary events.

**CRP and NF-κB.** Our study is the first to establish a link between CRP levels—NF-κB activation—and proinflammatory status in UA. It is also the first study to indicate that CRP, at concentrations commonly observed in UA, significantly induces NF-κB activation in circulating monocytes, amplifying the effects of a standard proinflammatory stim-
C-reactive protein at doses higher than the plasma concentration range observed in saphenous vein endothelial cells (29), but at doses markedly higher than the plasma concentration range observed in patients with ACS. C-reactive protein at doses >10 μg/ml also induces the expression of the chemokine IL-8, a powerful trigger of monocyte adhesion to endothelium, in human aortic endothelial cells and human coronary artery endothelial cells via up-regulation of NF-κB activity (30). Thus, the present data on circulating monocytes together with the available data on endothelial cells suggest that at least some of the detrimental effects of CRP promoting plaque instability might be mediated via the activation of the NF-κB signaling pathway.

Indeed, several effects of CRP, which has been found within atherosclerotic plaques (17–19), might directly contribute to plaque instability. First, the capacity of aggregated and ligand-complexed human CRP to activate the classic complement pathway and thus to express the major opsonic and chemotactic functions of the complement system has long been known (20,31). Second, bound CRP may be recognized by a subset of cellular Fc(γ) receptors (18) and thus directly activate phagocytic cells, as previously demonstrated for high concentrations of CRP (32,33) and confirmed in a previous study by our group for concentrations known to predict increased vascular event rates (9). Third, CRP has been reported to stimulate tissue factor production by peripheral blood monocytes and could thereby have important procoagulant effects (34,35). Fourth, CRP directly induces the expression of leukocyte adhesion molecules and of monocyte chemoattractant protein 1 by human endothelial cells, an inflammatory effect that can be inhibited by statins and PPAR-α activators (36,37). Finally, CRP has been shown to elicit several other detrimental effects on endothelial cells and/or vascular smooth muscle cells: it stimulates endothelin-1 and IL-6 production (38); it decreases PGI₂ and nitric oxide release (39,40); it up-regulates angiotensin type 1 receptor expression, thus promoting cell migration and proliferation in vitro and increasing reactive oxygen species production (41); and it inhibits endothelial progenitor cells differentiation, survival, and function, thus affecting key components of angiogenesis and response to chronic ischemia (42). Interestingly, CRP in atherosclerotic plaques is bound to low-density lipoproteins (18,31), and circulating ox-LDL have recently been shown to activate NF-κB in monocytes of UA patients (15).

Study limitations. Although potentially relevant for plaque destabilization, many of the claimed in vitro effects of CRP remain controversial because they might be caused by contaminants and/or additives in the CRP solution. Our findings may have similar limitations. However, differently from several previous studies, we used a number of strategies to ensure the specificity of CRP-mediated effects: the purity of CRP preparations was further confirmed by SDS/PAGE; CRP preparations were dialyzed twice to remove sodium azide; endotoxin was removed from CRP with a detoxigel column and all the reagents used were endotoxin free according to the limulus test; the cell cultures stimulated with CRP were coincubated with Polymixin B to ensure that CRP-induced effects were distinct from those mediated by LPS; all the effects on NF-κB disappeared when CRP denatured by incubation in boiling water bath for 1 hour was used; and 3 different methods were used to demonstrate CRP-induced NF-κB activation. Finally, although rhCRP was used for EMSA, the results obtained were confirmed by ELISA and confocal microscopy, where highly purified CRP isolated from human serum was used.

Clinical implications. We previously demonstrated that patients with a history of UA and persistently high levels of CRP represent a subset of patients with enhanced proinflammatory responsiveness of circulating leukocytes to a variety of potential stimuli (7–9); those patients are also at very high risk of recurrence of acute coronary events (3). The present study supports the notion that NF-κB activation might represent a mechanism by which raised levels of CRP amplify and perpetuate the inflammatory component of ACS and influence the clinical outcome. Indeed, our patients with persistent NF-κB activation had higher recurrence of acute coronary events during 12 months of follow-up than patients without NF-κB activation.

Overall, the present findings may have clinical implications, because PPAR-γ agonists or other inhibitors of NF-κB activation might result in new therapeutic approaches in ACS (43,44). The present data may also explain, at least partially, the beneficial effects of high-dose statins, because it has been consistently shown that high-dose statins improve the outcome of patients with ACS in association with a marked reduction of CRP levels (45,46).

Reprint requests and correspondence: Dr. Giovanna Liuzzo, Institute of Cardiology, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy. E-mail address: gliuzzo@hotmail.com.

REFERENCES


