# C-reactive protein activates the nuclear factor-κB signal transduction pathway in saphenous vein endothelial cells: Implications for atherosclerosis and restenosis

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**Objectives:** Elevated levels of C-reactive protein are one of the strongest prognostic factors in atherosclerosis. In addition to predicting vascular disease, C-reactive protein may directly facilitate the development of a proinflammatory and proatherosclerotic phenotype. Recent studies have demonstrated marked up-regulation of various adhesion molecules and inflammatory responses in endothelial cells subjected to C-reactive protein. The nuclear factor-κB signal transduction is known to play a key role in the expression of these proatherogenic entities. This study examines the direct effects of C-reactive protein on nuclear factor-κB activation and related mechanisms in saphenous vein endothelial cells.

**Methods:** The activation of nuclear factor-κB was determined by confocal microscopy assessing the nuclear localization of nuclear factor-κB in endothelial cells incubated with C-reactive protein (50 μg/mL) for 30 minutes and 3 hours. Cells not incubated with C-reactive protein were used as negative controls, and cells incubated with tumor necrosis factor-α (10 ng/mL) for 15 minutes were used as positive controls in all studies. The degradation of IκB-α and IκB-β was assessed by Western blotting of the cell lysates obtained from cells incubated with human recombinant C-reactive protein (50 μg/mL) for 15 minutes, 30 minutes, and 1 hour.

**Results:** Nuclear factor-κB nuclear translocation in endothelial cells increased significantly after 30 minutes of incubation with C-reactive protein ($P < .01$). Nuclear localization of nuclear factor-κB returned to baseline levels after 3 hours of incubation with C-reactive protein. Incubation with C-reactive protein resulted in degradation of IκB-α that was maximal at 30 minutes ($P < .05$). C-reactive protein showed no significant effect on IκB-β degradation.

**Conclusions:** These data demonstrate, for the first time, that C-reactive protein activates the nuclear factor-κB signal transduction pathway in endothelial cells. Degradation of IκB-α, but not IκB-β, seems to be the major pathway leading to nuclear factor-κB nuclear translocation and activation induced by C-reactive protein. These data support the concept that C-reactive protein, at concentrations known to predict diverse vascular insults, directly facilitates a proinflammatory and proatherosclerotic phenotype through activation of nuclear factor-κB. These data have important implications for saphenous vein atherosclerosis in patients with elevated C-reactive protein levels.

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The advances in basic science in the past few years have established a fundamental role for inflammation in mediating all stages of atherosclerosis from initiation through progression and ultimate thrombotic complications.[1] C-reactive protein (CRP) is one of the most powerful predictors of myocardial infarction, stroke, and vascular death currently known[2-9] and has recently been demonstrated to have a predictive value for cardiovascular events exceeding that of low-density lipoprotein (LDL)-cholesterol.[10] Recent studies further indicate that CRP is not only an inflammatory marker of atherosclerosis and coronary events but also a mediator of atherosclerosis, because it has been demonstrated to markedly up-regulate the expression of cell adhesion molecules and inflammatory responses related to atherosclerosis.[11-14]

Nuclear factor (NF)-$\kappa$B has been implicated as a key mediator of atherosclerosis.[15-17] Most proinflammatory genes expressed in endothelial cells (ECs) during the initial phase of lesion formation and in response to inflammatory mediators are dependent on NF-$\kappa$B activation.[18] Because CRP up-regulates the production of a number of endothelial adhesion molecules known to be transcriptionally activated by NF-$\kappa$B,[13,19,20] this study investigated the direct effects of human recombinant CRP on NF-$\kappa$B activation and related mechanisms.

## Methods

### Cell Culture

Human saphenous vein ECs were harvested from vein segments obtained from patients undergoing bypass surgery and were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. ECs were plated onto 60-mm plates and grown to confluence before treatment. ECs between passages 2 and 4 were used for the studies outlined next. Human recombinant CRP (Calbiochem, San Diego, Calif) was used in all studies described. Given the concern surrounding the potential contamination of CRP with endotoxin, a number of control experiments were performed as described previously,[12] and all studies were performed in the presence of polymyxin-B (50 $\mu$g/mL, Sigma-Aldrich, St Louis, Mo).

### Confocal Microscopy

The activation of NF-$\kappa$B was determined by confocal microscopy assessing the nuclear localization of NF-$\kappa$B in cells incubated with CRP (50 $\mu$g/mL) for 30 minutes and 3 hours. Cells not incubated with CRP were used as negative controls, and cells incubated with tumor necrosis factor (TNF)-$\alpha$ (10 ng/mL, Sigma-Aldrich) for 15 minutes were used as positive controls in all studies. Cells were stained using a 1:500 dilution of primary antibody for p65 (sc-372, Santa Cruz Biotechnology Inc, Santa Cruz, Calif), a subunit of NF-$\kappa$B, which is abundant in the cytoplasm. Tyramide signal amplification (NEN Life Science Products, Boston, Mass) was used to detect p65 according to the manufacturer's protocol. Primary antibody incubation was for 2 hours at room temperature. Subsequent incubations were biotin-conjugated secondary antibody for 30 minutes, streptavidin-conjugated horseradish peroxidase for 30 minutes, and fluorescein isothiocyanate-conjugated tyramide complex for 8 minutes. Tris-HCl blocking buffer (NEN) was used for diluting antibodies and washes. Nuclei were counterstained with propidium iodide (Sigma-Aldrich).

Images of the ECs were taken using a Bio-Rad MRC-1024ES confocal microscopy (Hercules, Calif) with a krypton and argon laser. In every experimental group, 5 images were obtained from different regions of the stained slides. Confocal settings were optimized and maintained for all images obtained. Fluorescent staining and NF-$\kappa$B nuclear translocation were quantified using pixel distribution data from the confocal images to indicate the percentage of pixels positive for both p65 and nuclear stain to indicate the percentage of nuclei with translocated p65.

### Western Blotting

The effects of CRP on the degradation of I$\kappa$B-$\alpha$ and I$\kappa$B-$\beta$ were determined by Western blotting with the use of I$\kappa$B-$\alpha$ and I$\kappa$B-$\beta$ polyclonal antibodies (sc-1643 and sc-945, Santa Cruz Biotechnology Inc). Briefly, EC lysates obtained from cells incubated with CRP (50 $\mu$g/mL) for 15 minutes, 30 minutes, and 1 hour or with TNF-$\alpha$ (10 ng/mL) for 15 minutes were fractionated through a 4% stacking and 10% running sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and the fractionated proteins were transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 hour at room temperature with blocking buffer (5% nonfat milk in 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20). Rabbit anti-angiotensin II receptor type 1 and type 2 polyclonal immunoglobulin Gs (Transduction Laboratories, Lexington, Ky), at a dilution of 1:200, were reacted with the blots overnight at 4°C. After washing 2× for 15 minutes in 1× TTBS (Tris-buffered saline with 0.05% Tween 20), the blots were incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (Santa Cruz Biotechnology Inc) at 1:2000 dilution for 1 hour at room temperature. Visualization was performed with the use of enhanced chemiluminescence. Densitometric analysis of Western blots was performed with the use of PDI Imageware System (San Diego, Calif).

### Statistical Analysis

The results are presented as mean ± SD. Statistical comparisons were performed by analysis of variance followed by Dunn's multiple contrast hypothesis test to identify differences between various treatments or by the Mann-Whitney *U* test.

## Results

### Activation of NF-$\kappa$B

At baseline, ECs stained for p65 showed an abundance of this NF-$\kappa$B subunit in the cytoplasm (Figure 1, *A*). After ECs were stimulated with human recombinant CRP (50 $\mu$g/mL) for 30 minutes, p65 nuclear translocation significantly increased by 4.5-fold ($P < .01$) and then returned to baseline after 3 hours, indicating an early phase activation

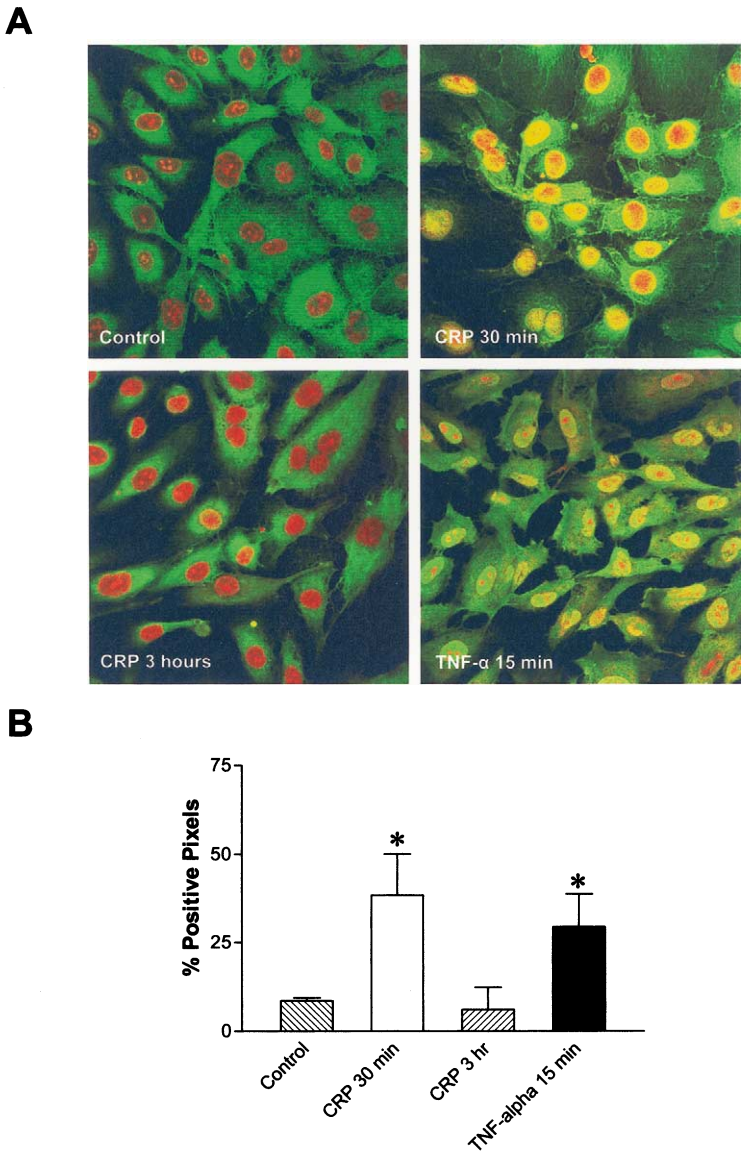


**Figure 1. A, Representative immunoconfocal images of control ECs. ECs stimulated with human recombinant CRP (50 $\mu$g/mL) for 30 minutes and 3 hours or the positive control, TNF-$\alpha$ (10 ng/mL), for 15 minutes. Cells were stained for p65, a subunit of NF-$\kappa$B (*green*), which is abundant in the cytoplasm. Nuclei were counterstained with propidium iodide. Increased p65 nuclear translocation occurs with cells stimulated with CRP for 30 minutes, and this response disappears by 3 hours. B, Fluorescent staining is quantified using pixel distribution data from confocal images to indicate the percentage of pixels positive for both p65 and nuclear stain (propidium iodide) to indicate the percentage of nuclei with translocated p65 (n = 5). (*$P < .05$ different from control.) *CRP,* C-reactive protein; *TNF,* tumor necrosis factor.**

of NF-$\kappa$B in these cells (Figure 1). The positive control, TNF-$\alpha$ (10 ng/mL) stimulation for 15 minutes, induced a 3.5-fold increase in p65 nuclear translocation ($P < .05$ compared with the negative control) (Figure 1).

### Western Blotting of I$\kappa$B-$\alpha$ and I$\kappa$B-$\beta$

Incubation with CRP (50 $\mu$g/mL) resulted in an increased degradation of I$\kappa$B-$\alpha$ that was first observed at 15 minutes and was maximal at 30 minutes ($P < .05$) (Figure 2, *A*). This effect of CRP slightly decreased at 1 hour. In the positive control group, TNF-$\alpha$ significantly increased the degradation of I$\kappa$B-$\alpha$ at 15 minutes (Figure 2, *A*). CRP showed no significant effect on I$\kappa$B-$\beta$ degradation; however, TNF-$\alpha$ (10 ng/mL) significantly increased the degradation of I$\kappa$B-$\beta$ ($P < .01$) (Figure 2, *B*).

## Discussion

During the past few years we have witnessed a paradigm shift in our understanding of the underlying principles of atherosclerosis. This "new view" supports the concept that vascular inflammation is the central orchestrator of atherosclerotic lesion formation, progression, and eventual rupture.[1] This paradigm shift has fueled exponentially increasing interest in evaluating inflammatory markers of atherosclerosis, of which high-sensitivity CRP has emerged as one of the most important.[2-7] As such, the inflammatory marker CRP is one of the most powerful independent predictors of myocardial infarction, stroke, and vascular death in a variety of settings. This acute-phase reactant has also been shown to predict future coronary events and portend the vulnerability of an atherosclerotic lesion and the likelihood of plaque rupture. During the past year, much interest has been generated in unraveling the mechanistic basis of the CRP and atherosclerosis connection. Indeed, recent studies, including work from our group, suggest that CRP is not only a predictor but also an active partaker in lesion formation.[11-14,19,20] CRP, at concentrations known to predict vascular disease, has a direct effect on stimulating diverse early atherosclerotic processes including EC adhesion molecule expression, chemoattractant chemokine secretion, and macrophage LDL uptake.[13,19,20] At the level of vascular smooth muscle, CRP directly stimulates the angiotensin-type 1 receptor and promotes smooth muscle cell proliferation, migration, and neointimal formation in vitro and in vivo.[14] Thus, CRP is not only an inflammatory marker of atherosclerosis and coronary events but also a mediator of the disease, because it contributes to the substrate underlying lesion formation, plaque rupture, and coronary thrombosis through interaction with and alteration of the vascular phenotype.

NF-$\kappa$B has been implicated as a key mediator of atherosclerosis.[15-17] This transcription factor is a DNA binding protein complex that is usually present in the cytosol as an inactive complex. I$\kappa$B, an associated protein, renders this complex inactive by shielding the nuclear localization signal.[21] Although several inhibitor proteins have been identified (I$\kappa$B-$\alpha$, I$\kappa$B-$\beta$, I$\kappa$B-$\gamma$, and p105), I$\kappa$B-$\alpha$ is the best characterized form of I$\kappa$B.[16] On I$\kappa$B phosphorylation and its subsequent degradation, the heterodimeric NF-$\kappa$B complex translocates from the cytoplasm to the nucleus, where it binds to specific DNA sequences in the promoter region of several genes and up-regulates their transcription. Multiple genes whose products are putatively involved in the atherosclerotic process are regulated by NF-$\kappa$B.[18] Transcriptional activation of the genes encoding the cell adhesion molecules (vascular cell adhesion molecule-1, intracellular adhesion molecule-1, E-selectin) and chemokines (monocyte chemoattractant protein-1) are tightly regulated by the transcription factor NF-$\kappa$B. The present study demonstrates that CRP directly increases the degradation of I$\kappa$B-$\alpha$ and subsequently activates the NF-$\kappa$B pathway.

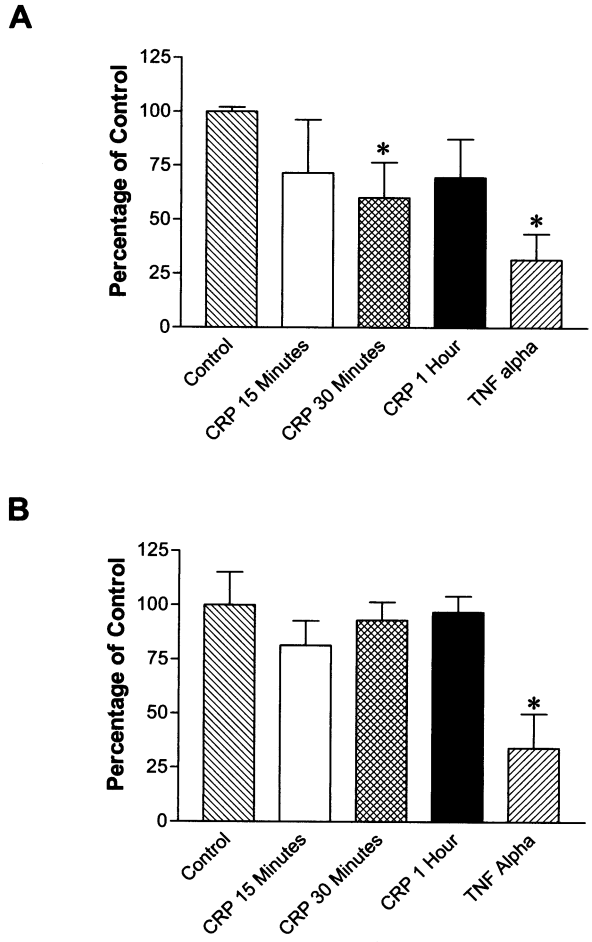


**Figure 2. I$\kappa$B-$\alpha$ (A) and I$\kappa$B-$\beta$ (B) protein levels in unstimulated control human saphenous vein ECs. Cells stimulated with human recombinant CRP (50 $\mu$g/mL) for 15 minutes, 30 minutes, and 1 hour or the positive control, TNF-$\alpha$ (10 ng/mL), for 15 minutes (n = 5). Protein levels were assessed by Western blotting of cell lysate. (*$P < .05$ different from control.)**

Activation of NF-$\kappa$B has been linked to protection against apoptosis in certain circumstances.[22-24] However, CRP has been shown to facilitate EC apoptosis.[12] The findings in the present study showed that increased p65 nuclear translocation rapidly and transiently occurred with ECs stimulated with CRP for 30 minutes, and that this response disappeared by 3 hours. This transient increase of NF-$\kappa$B likely activates the expression of a number of the proatherogenic factors but does not have protective effects on EC apoptosis during later phases.

These data demonstrate, for the first time, that CRP directly activates the NF-$\kappa$B signal transduction pathway in saphenous vein ECs. Degradation of I$\kappa$B-$\alpha$, but not I$\kappa$B-$\beta$, seems to be the major pathway leading to NF-$\kappa$B nuclear

localization and activation induced by CRP. These data support the growing body of evidence indicating that CRP is not only a marker but also an active mediator of atherosclerotic lesion formation and that patients with elevated levels of CRP may be at increased risk of saphenous vein atherosclerosis.

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## Discussion

**Dr Alexander Wahba** *(Trondheim, Norway).* Do you know why some patients would have a high CRP compared with other patients? What would your target be, and how would you try to treat this?

**Dr Verma.** It is unclear what the receptor is on the endothelium that mediates all of these pleiotropic effects of CRP. We have performed some preliminary work with this, and we suggest that it is the LOX1 (Lectin-like oxidized low-density lipoprotein receptor 1) receptor, the receptor that was originally defined to bind oxidized LDL. The LOX1 receptor now seems to bind a number of structurally distinct molecules, including apoptotic cells, and some preliminary data from our group indicate that CRP strongly binds to LOX1 and that LOX1 antagonists block the proatherogenic effects of CRP.

**Dr Wahba.** What is the cause for having a high CRP?

**Dr Verma.** The cause for an elevated CRP usually reflects underlying inflammation and is a surrogate for the underlying atherosclerotic process. Risk factors, both genetic and acquired, influence CRP, as does ethnicity.

**Dr Carmelo A. Milano** *(Durham, NC).* That was an excellent presentation and a lot of data. I was wondering if you could speak a little bit about the dose of CRP you used in these in vitro studies. Do you have a mechanism by which you can correlate that to what CRP levels might be in vivo in a patient or an animal?

**Dr Verma.** We have looked at CRP concentrations from 1 to 100 $\mu$g/mL, and the reason for using those CRP concentrations is because those are really the values that seem to predict future cardiovascular events.

**Dr Jakob Vinten-Johansen** *(Atlanta, Ga).* That was a beautiful study. It really should leave something for the rest of us to do.

Have you tried the concept in vivo? Have you looked at whether CRP, for example, increases neutrophil adherence, migration, and so forth, and then have you tried it in an in vivo saphenous vein model graft?

**Dr Verma.** We have performed in vivo carotid angioplasty experiments in the presence and absence of continuous CRP administration and demonstrated at a 4-week time point that there

was increased vascular smooth muscle cell migration and proliferation by bromodeoxyuridine staining. That actually correlates very nicely with increased intimal medial thickness and angiotensin-1 receptor density.

The reason why we cannot perform experiments exceeding 4 weeks in rats is the way we perform the experiments. We give 2 local injections of CRP at the site and then use a gelling agarose gel to keep the CRP at the site of injury, and what we have noticed is that after 6 weeks we start losing that effect; thus, we chose the 4-week time point to mimic a long-term model of high CRP.

**Dr Y. Joseph Woo** *(Philadelphia, Pa).* That was a great study. You postulated several different mechanisms. Would you elaborate on the potential role for the increased elimination of the endothelial progenitor cells and what that might have to do with the overall mechanism?

**Dr Verma.** I think there are 2 mechanisms operating here. First, there are direct proatherogenic effects that promote not only atherosclerosis at the level of the endothelium but also neointimal hyperplasia at the level of the vascular smooth muscle, and we believe that the unifying hypothesis is mediated through LOX1.

The effects of CRP on endothelial progenitor cells are probably a completely distinct mechanism through which CRP not only promotes atherosclerosis but also inhibits the compensatory mechanism in atherosclerosis (ie, the recruitment and homing of bone marrow-derived progenitor cells inhibiting angiogenesis).