C-Reactive Protein Alters Antioxidant Defenses and Promotes Apoptosis in Endothelial Progenitor Cells

Hiroko Fujii, Shu-Hong Li, Paul E. Szmitko, Paul W.M. Fedak, Subodh Verma

Objective—C-reactive protein (CRP) has been suggested to participate in the development of atherosclerosis, in part, by promoting endothelial dysfunction and impairing endothelial progenitor cell (EPC) survival and differentiation. In the present study, we evaluated the effects of CRP on antioxidative enzymes, reactive oxygen species production, telomerase activity, and apoptosis in human circulating EPCs.

Methods and Results—EPCs, isolated from peripheral venous blood, were cultured in the absence or presence of native pentameric azide and lipopolysaccharide (LPS)-free CRP (0, 5, 15, and 20 µg/mL), N-acetylcysteine (NAC), hydrogen peroxide (H$_2$O$_2$) or monoclonal anti-CRP antibodies. Fluorescence-activated cell sorter (FACS) analysis was used for the measurement of intracellular H$_2$O$_2$ and superoxide (O$_2^-$) by loading cells with 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA). Apoptosis was evaluated with Annexin V immunostaining and cytosolic cytochrome c expression. Western blot analysis was used for the determination of manganese superoxide dismutase (MnSOD) and glutathione peroxidase expression, and polymerase chain reaction enzyme-linked immunosorbent assay was used to assess telomerase activity. Incubation of EPCs with CRP caused a concentration dependent increase in reactive oxygen species (ROS) production and apoptosis, with an effect quantitatively similar to H$_2$O$_2$. This effect was attenuated during coincubation with NAC or anti-CRP antibodies. Furthermore, CRP altered EPC antioxidative enzyme levels, demonstrating a reduced expression of glutathione peroxidase and a significant increase in MnSOD expression. Transfection of EPCs with MnSOD-RNAi resulted in a reduction in CRP-induced ROS production, apoptosis, and telomerase inactivation.

Conclusions—CRP, at concentrations known to predict cardiovascular events, may serve to impair EPC antioxidant defenses, and promote EPC sensitivity toward oxidant-mediated apoptosis and telomerase inactivation. These data further support a direct role of CRP in the development and/or progression of atherothrombosis. (Arterioscler Thromb Vasc Biol. 2006;26:2476-2482.)

Key Words: antioxidative enzymes ■ apoptosis ■ C-reactive protein ■ endothelial progenitor cells ■ reactive oxygen species

Cardiovascular diseases, including diabetes and atherosclerosis, are typically characterized by elevated levels of reactive oxygen species (ROS), endothelial dysfunction and proinflammatory states. Classically considered as part of the innate immune system, CRP functions to activate the complement system, induce adhesion molecule expression, enhance macrophage phagocytosis, and promote leukocyte activation. Importantly, CRP may promote endothelial dysfunction via effects to decrease endothelial nitric oxide synthase (eNOS) expression and mRNA stability, in addition to effects to promote endothelial LOX-1 expression, stimulate ROS production, and promote endothelial apoptosis. Striking evidence for a direct effect of CRP on endothelial function is provided by Stroes et al who demonstrated an effect of CRP infusion to directly attenuate vascular function in humans. Furthermore, CRP also inhibits bone-marrow derived endothelial progenitor cell (EPC) survival and differentiation.

EPCs are bone marrow derived stem cells that have the ability to differentiate into functional, mature endothelial cells. EPCs circulate in the blood and appear to home preferentially to sites of vascular or tissue injury, contributing significantly to both re-endothelialization and neovascularization. Part of the regenerative capacity of EPCs appears to be attributed to their high expression of antioxidative proteins, low baseline ROS levels, and reduced sensitivity toward ROS-induced cell death, which likely also leads to the maintenance of telomerase activity and the prevention of replicative senescence and cellular dysfunction.
more, telomerase reverse transcriptase (TERT) activity, which is lost with elevated intracellular ROS production, contributes to EPC angiogenic properties. Increased generation of ROS stimulates the export of TERT from the nucleus into the cytoplasm, resulting in cell aging.

Previously, our group demonstrated that CRP directly inhibits EPC differentiation, survival, and function, in part via CRP’s ability to reduce EPC eNOS expression. In the present study, we tested the effects of LPS and azide-free CRP, at concentrations known to predict adverse cardiac events, on ROS production in EPCs, its influence on antioxidative enzyme expression, and its effect on telomerase activity and apoptotic cell death. Furthermore, we evaluated the potentially protective effect of N-acetylcysteine on EPCs under oxidative stress.

Materials and Methods

Cell Culture

Mononuclear cells were isolated from the peripheral blood of healthy human volunteers using the Vacutainer Cell Preparation Tube system for the separation of mononuclear cells from whole blood (Becton Dickinson) as per the manufacturer’s directions. Immediately after isolation, the mononuclear cells (10⁶ cells/ml) were plated on culture dishes coated with human fibronectin and maintained in endothelial basal medium-2 (EBM-2; Clonetics) supplemented with EGM SingleQuots and 20% fetal bovine serum. Purified human endothelial basal medium-2 (EBM-2; Clonetics) supplemented with EGM SingleQuots and 20% fetal bovine serum. Purified human Factor 8 (Sigma). The presence of antibody was confirmed by the demonstration of increased cytosolic cytochrome c expression (Figure 2). Likewise, with increasing concentrations of CRP, EPCs underwent necrotic cell death as noted by PI staining (supplemental Figure I).

Results

Exposure to CRP Increases the Formation of ROS in EPCs and Promotes Cell Apoptosis and Necrosis

ROS formation was measured by the detection of H₂DCF-DA via flow cytometry. Compared with the control group, treatment of EPCs with either 5 μg/mL or 15 μg/mL of sodium azide and LPS-free human CRP resulted in a significant increase in the generation of ROS in a dose dependent fashion (Figure 1A). MitoQ is a highly selective scavenger of intramitochondrial generated ROS and we used this to evaluate whether the source of EPC ROS production by CRP could be ascribed to a mitochondrial origin. Thirty minutes of MitoQ pretreatment (10 μmol/L) prevented CRP-induced increases in EPC ROS production (Figure 1B). Corresponding to this increase in ROS formation, treatment with CRP resulted in a concentration-dependent increase in both EPC cell death via apoptosis and necrosis. Using Annexin V staining, there was a difference in the extent of apoptosis among the control and CRP-treated EPCs (Figure 2). Although a CRP concentration of 5 μg/mL failed to result in a significant increase in EPC apoptosis, at concentrations of greater than 15 μg/mL, CRP promoted apoptotic cell death (P<0.001). The effect of CRP on EPC apoptosis was further confirmed by the demonstration of increased cytosolic cytochrome c expression (Figure 2). Likewise, with increasing concentrations of CRP, EPCs underwent necrotic cell death as noted by PI staining (supplemental Figure I).

Pretreatment With Either Anti-CRP Antibodies or N-acetylcysteine Impairs CRP-Induced ROS Formation in EPCs and Protects EPCs From Apoptotic Cell Death

To investigate whether the CRP-induced increase in ROS formation causally contributes to EPC death, CRP-treated EPCs were incubated with both the antioxidant N-acetylcysteine and antibodies to CRP. The extent of ROS formation was once again assessed by detection of H₂DCF-DA. CRP-induced ROS formation in EPCs was significantly reduced by pretreatment with either NAC or anti-CRP antibodies compared with ROS generation in EPCs treated with CRP at a concentration of 15 μg/mL (Figure 3). This reduction in ROS formation appeared to translate into a reduction in EPC apoptosis as assessed by the extent of Annexin V cell staining. Pretreatment with NAC significantly reduced the extent of CRP-induced apoptotic cell death, comparable to the extent of protection afforded by NAC pretreatment of EPCs exposed to H₂O₂ (Figure 3).

Please see the online data supplement for additional details on Materials and Methods (http://atvb.ahajournals.org).
CRP Alters the Expression of Antioxidative Enzymes in EPCs in a Manner That Promotes ROS Production

Previously, it was shown that EPCs have greater levels of antioxidative enzyme expression (compared with endothelial cells) providing protection against oxidative stress and thereby enhancing their progenitor function. Among these antioxidative enzymes are MnSOD and glutathione peroxidase. To examine whether CRP interferes with the expression of these antioxidative enzymes in EPCs, EPCs were incubated with CRP and the extent of MnSOD and glutathione peroxidase expression was elucidated by Western blot analysis. As shown in Figure 4, CRP treatment caused a significant decrease in glutathione peroxidase but a significant increase in MnSOD protein expression in EPCs.

To address the role of MnSOD in CRP treated EPCs, we transfected EPCs with MnSOD-RNAi or scrambled RNAi. The extent of MnSOD expression in EPCs transfected with MnSOD-RNAi was, as expected, significantly decreased (Figure 5), and thus the production of ROS in these EPCs was increased compared with those without MnSOD-RNAi and only scrambled RNAi (Figure 5). Next, we examined the formation of ROS in EPCs transfected with MnSOD-RNAi or scrambled RNAi and treated with CRP (15 μg/mL). In EPCs with MnSOD-RNAi, ROS production was increased, because there were decreased levels of antioxidative enzymes. However, in EPCs with MnSOD-RNAi and treated with CRP, the extent of ROS production was decreased compared with CRP-treated EPCs with only scrambled RNAi (Figure 5). Furthermore, MnSOD-RNAi attenuated CRP-induced EPC apoptosis (Figure 5). Lastly, CRP significantly reduced EPC telomerase activity, a response that was attenuated by MnSOD-RNAi (Figure 6). These data suggest a central role of MnSOD as a mediator of CRP-induced oxidative apoptosis and EPC senescence.

Discussion

The present study uncovers yet another biological mechanism through which CRP promotes endothelial dysfunction and sets the stage for atherosclerosis. By promoting the generation of ROS by altering the homeostatic balance of antioxidative enzymes in EPCs, CRP facilitates both apoptotic and necrotic cell death. Furthermore, in addition to this detrimental effect on EPC survival, the CRP-mediated reduction in ROS formation is impaired by pretreatment with N-acetylcysteine (NAC) or anti-CRP antibodies.
Atherosclerosis is currently regarded as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation. Human recombinant CRP has been demonstrated to elicit a multitude of effects on endothelial biology favoring a proinflammatory and proatherosclerotic phenotype. In vitro experiments reveal that CRP potently downregulates eNOS transcription and destabilizes eNOS mRNA, resulting in decreased basal and stimulated nitric oxide release. Simultaneously, CRP stimulates endothelin-1 and interleukin-6 release from endothelial cells, and decreases the production of the potent vasodilator prostacyclin, shifting the balance further toward endothelial dysfunction. In addition to these effects on endothelial activation, CRP has been linked to thrombosis, via direct effects on plasminogen activator inhibitor-1 and tissue plasminogen activator bioavailability. It is hypothesized that CRP mediates its biological effects on endothelial cells by binding and becoming internalized through Fcγ receptors, CD32 and CD64, and is also believed to be released locally, from endothelial cells. Growing evidence suggests that bone marrow-derived EPCs circulate in the blood and play an important role in the formation of new blood vessels as well as contribute to vascular homeostasis in the adult. Numerous animal studies have shown that EPCs can integrate into new and existing blood vessels and repair areas of endothelial damage. Clinical studies suggest that traditional risk factors for coronary atherosclerosis are associated with lower levels of circulating EPCs and that the number of these progenitor cells in the circulation can be used to predict the occurrence of cardiovascular events and death. Our group has previously demonstrated the adverse effect CRP has on EPC biology. The current study provides further insight into how CRP may negatively affect EPCs by quenching antioxidant defenses and promoting telomerase inactivation.

There has been recent controversy surrounding the proinflammatory effects of CRP on endothelial cells, with suggestion that these effects may be secondary to contaminants in...
commercially available CRP preparations, including sodium azide and LPS.27,28 The balance of published evidence, as recently reviewed, strongly supports a direct role of CRP in atherothrombosis, independent of contamination with azide or LPS.5,21 For example, endotoxin-purified, azide free CRP inhibits nitric oxide release, and decreases tPA in aortic endothelial cells.21 The role of LPS and sodium azide toward the effects of CRP on endothelial activation have been laid to rest by observations in endothelial cells indicating that: (1) monoclonal antibodies to CD32 inhibit the proinflammatory effects of CRP; (2) LPS (50 and 1000 pg/mL) had no effect on CRP induced tissue plasminogen activator activity; (3) polymyxin B did not affect CRP’s effects, whereas boiling and/or trypsinization abrogated the effect; and (4) the proinflammatory effects of CRP were observed in cells that had been preabsorbed to anti-CRP IgG-coated plates but not to plates without anti-CRP IgG. These observations argue strongly in favor of a specific proatherosclerotic effect of CRP, not attributable to contaminants or sodium azide. In addition to using sodium azide free CRP from Trichem, we dialyzed CRP 3 times, with no change observed in the effects of either native or monoclonic CRP on EPC ROS expression.

Furthermore, monoclonal antibodies to CRP inhibited the effects of CRP on endothelial cells and EPCs. Also recently, Montero et al.10 report that the effects of CRP on matrix metalloproteinase (MMP)-1 and MMP-10 are specific to CRP and not mediated by endotoxin and azide which failed to have any effects on MMP secretion. Finally, Stroes et al demonstrated a direct effect of CRP on endothelial activation and inflammation after infusion in humans,10 an effect that cannot be attributed to LPS or azide contamination.29 A direct role of CRP in atherothrombosis is suggested by recent work from Dr Pepy’s group wherein a direct CRP inhibitor afforded protection against experimental myocardial infarction.35 The effects of CRP on endothelial cells are specific and receptor-mediated events, and not caused by commercial contamination.

CRP, in a dose-dependent fashion, caused the generation of ROS in EPCs. ROS such as the superoxide radical, H2O2 and the hydroxyl radical, may be produced in vascular cells in response to growth factors such as platelet-derived growth factor and granulocyte macrophage-colony stimulating factor.30 These ROS can then act as potent second messengers, triggering signal transduction pathways which regulate cell growth, aging, and transformation.31 However, when the rate of ROS formation exceeds the capacity of the antioxidative defenses, a state of oxidative stress occurs, which can lead to cell death.32 EPCs have been shown to exhibit a significantly lower basal ROS concentration as compared with mature endothelial cells, to have higher levels of antioxidative enzyme expression, and thus to be more resistant to oxidative stress in keeping with their progenitor cell character.15 However, treatment with CRP causes EPCs to partially lose this resistance to oxidative stress by altering the balance of antioxidative enzymes, forcing them into early senescence.

CRP significantly altered the expression of the antioxidative enzymes glutathione peroxidase and MnSOD; however, in opposite directions. Levels of glutathione peroxidase were decreased, and thus ROS levels should be increased. In contrast, levels of MnSOD expression in EPCs were increased by CRP. However, higher levels of MnSOD expression do not necessarily result in antioxidative protection. As observed in the current study, when EPCs transfected with MnSOD-RNAi, and thus, inhibited MnSOD function, were treated with CRP, the extent of ROS generation was diminished. Previous reports suggest that overexpression of MnSOD results in cell growth and sensitization to oxidative damage rather than antioxidative protection.33 The mechanism behind this observed effect is believed to involve MnSOD induced overproduction of H2O2. MnSOD serves as one of the initial antioxidant enzymes that convert superoxide radicals to H2O2 in the mitochondria. Glutathione peroxidase and catalase then subsequently detoxify H2O2 to water.34 Thus, by inducing MnSOD expression and down regulating glutathione peroxidase expression, an excessive amount of H2O2 will be produced that cannot be adequately detoxified resulting in H2O2 induced EPC death. This is further demonstrated by the MnSOD-RNAi studies. CRP treated EPCs that were transfected with the MnSOD-RNAi would not generate excessive amounts of H2O2 and thus, demonstrated reduced ROS generation.

Finally, we show that CRP enhanced ROS production also resulted in a significant reduction of TERT activity. ROS appear to stimulate the export of TERT from the nucleus into the cytosol, which may lead to a loss in the ability to prolong telomeres, resulting in progressive telomere shortening, reduced replicative ability and increased sensitivity toward apoptotic stimuli.16 Because telomerase is capable of countering the onset of cellular senescence, it is thus an important component of the regenerative properties of EPCs in neovascularization and vascular repair. The overexpression of human TERT in EPCs prevented downregulation of eNOS, improved the functional activity of EPCs for vascular regeneration, and enhanced EPC survival.17 Thus, by inactivating telomerase activity, CRP impairs an important molecular component of EPC survival and ongoing regenerative ability.

Figure 6. CRP reduces telomerase activity in EPCs. Telomerase activity was assessed in EPCs transfected with scrambled RNAi or with MnSOD-RNAi and treated with or without CRP (15 μg/mL). CRP promoted telomerase inactivation in EPCs transfected with scrambled RNAi. This effect was attenuated by MnSOD-RNAi treatment. (P<0.05 vs other groups).
The role of CRP as a partaker of cardiovascular disease continues to evolve, and is strengthened by recent studies that demonstrate that endothelial cells produce CRP, and that CRP promotes leukocyte–endothelial interactions. Additional evidence for an in vivo role of CRP in cardiovascular disease stem from studies demonstrating the ability of CRP to induce myocardial infarction in a rat coronary ligation model, increased susceptibility to simulated cardiomyocyte ischemia and reperfusion (Verma unpublished observations) and increased cerebral infarct size in rats following middle cerebral artery occlusion, and promote neointimal formation after balloon angioplasty in a rat model. In the hypercholesterolemic pig model, Turk et al showed that serum CRP correlated with macrophage accumulation and coronary artery disease, immunohistochemically they demonstrated costaining for CRP in the macrophage foam cells in the intima. Recently, Sun et al measured CRP levels in the plasma of hypercholesterolemic rabbits and investigated CRP expression at both the mRNA and protein levels using rabbit and human atherosclerotic specimens. CRP levels were significantly elevated in both cholesterol-fed and Watanabe-heritable hyperlipidemic rabbits, and CRP levels were clearly correlated with aortic atherosclerotic lesion size. Immunohistochemical staining coupled with Western blotting analysis revealed that CRP-immunoreactive proteins were found at all stages of atherosclerosis from the early to advanced lesions. CRP was present extracellularly and colocalized with apolipoprotein B but was rarely associated with the cytoplasm of macrophages and foam cells further pointing to a role of CRP in the pathophysiology of atherothrombosis. As stated by Torzewski, it is important to note that the most broadly available animal model, ie, the mouse, is considered useless regarding the study of CRP functions because CRP is not an acute phase reactant in mice. CRP is a foreign antigen in the mouse with many uncertainties concerning its functional role in the immune system of these animals. The fact that CRP transgenic mice are incapable of mounting one of the most fundamental effects of CRP, ie, lipoprotein-dependent complement activation argue strongly against the validity of this model for physiological and pathophysiological assessments.

Taken together, the data of the present study reveal that native, pentameric CRP, free of azide or LPS, directly induces ROS formation in EPCs by altering the expression of antioxidative enzymes. This elevation in ROS formation subsequently leads to diminished telomerase activity and, ultimately, EPC death. In this fashion, CRP may impair progenitor-mediated endothelial repair, contributing further to the development and progression of atherothrombosis.

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Disclosures
None.

References

Fujii et al  CRP and EPC Oxidative Stress


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Detection of Oxidative Stress

After 7 days in culture, living cells were incubated for dye uptake with 5 \( \mu \text{mol}/L \) of 2',7'-dichlorodihydrofluorescein diacetate (H\(_2\)DCF-DA; Molecular Probes) for 30 minutes in the dark. The cells were then washed once with PBS, trypsinized for 2 minutes, and the reaction stopped with PBS containing 10% FBS. The cells from each group were then analyzed for ROS by flow cytometry. For groups pretreated with either NAC or anti-CRP antibodies, pretreatment was performed on cells in the 7\(^{th}\) day of culture, 30 minutes prior to CRP treatment. EPCs were incubated with 100 \( \mu \text{mol}/L \) NAC or anti-CRP antibody (1:6000) 30 minutes prior to CRP treatment. 3 hours later, the cells were harvested and analyzed via flow cytometry. Experiments were also performed with 30 minute pre-treatment with MitoQ (10\( \mu \text{M} \)) to inhibit mitochondrial ROS generation.

Contd…
Online Supplementary Material

**Apoptosis Assay**

To assess the extent of EPC apoptosis, fluorescence-labeled Annexin-V-FLUOS staining was performed according to the manufacturer’s instructions (Roche Molecular Biochemicals). Additionally, the effects of CRP on cytosolic cytochrome c was studied as a biomarker of apoptosis. After treatment of CRP, the cells were collected and lysed with lysis buffer (20 mmol/l HEPES/NaOH, pH 7.5, 250 mmol/l sucrose, 10 mmol/l KCl, 2 mmol/l MgCl₂, 1 mmol/l EDTA, 1 mmol/l DTT, protease inhibitor cocktail) for 20 min on ice. The samples were homogenized by 10 passages through a 2-gauge needle. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. Western blot analysis for cytochrome c was performed. Necrotic cells were assessed via uptake of propidium iodide (PI). Cell groups were treated with NAC and CRP as described above. For the H₂O₂ groups, cells were pretreated 30 minutes prior to H₂O₂ treatment with NAC (100 μmol/L) on day 6 of culture, with cells being analyzed 18 hours post H₂O₂ exposure. Cells were visualized using dual-emission confocal microscopy.

**Western Blot Analysis**

For determination of manganese superoxide dismutase (MnSOD) and glutathione-peroxidase expression, EPCs were lysed with 200 μL of cell lysis buffer (20 mmol/L Tris (pH 7.5), 150 mmol/mL NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerolphosphate, 1 mmol/L Na₃VO₄, 1 μg/mL leupeptin, and 1 mmol/L phenylmethyisulfonyl fluoride) on ice for 15 minutes. The cells were harvested and centrifuged, with the resulting cell pellet resuspended in 50 μl of cell lysis buffer with 1% triton and incubated on ice for 5 min. The suspended cells were then sonicated for 5 seconds on four occasions and then centrifuged at 10 000g for 1 minute. The supernatant containing cellular protein was taken and stored at −80°C prior to use. Proteins (10 μg per lane) were loaded onto SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Western blots were performed by using primary antibodies directed against MnSOD (1:1000; BD Biosciences), and glutathione-peroxidase (1:100;
Calbiochem). Enhanced chemiluminescence was performed using goat anti-mouse IgG-HRP (1:2000) and goat anti-rabbit IgG-HRP (12000) as secondary antibodies. The resulting films were scanned and semiquantitatively analyzed.

**Telomerase Enzyme Activity Measurement**

Telomerase enzyme activity was measured using the TeloTAGGG Telomerase PCR ELISA PLUS assay according to the manufacturer’s protocol (Roche Diagnosis Corporation).

**RNA-interference (RNAi)**

MnSOD-RNA-interference (RNAi) (5’ CCA CGA TCG TTA TGC TGA GTT 3’) and scrambled RNAi (5’ AAT CTC GTG TCG TGA CAC GTT 3’), were used to evaluate the function of MnSOD (Qiagen Inc.). On the day prior to transfection, 5 x 10⁵ cells were plated per well, on a 6 well plate, in 2 mL of EBM-2. Cells were incubated for 24 hours prior to transfection. On the day of transfection, 5 μg of iRNA was diluted in the appropriate volume of culture medium to give a final volume of 100 μL. For complex formation, 15 μL of RNAiFect Transfection Reagent were added to the diluted iRNA and the samples were incubated for 15 minutes at room temperature. While complex formation was taking place, the medium was removed from the culture plates, and 1885 μL of fresh cell growth medium were added to the cells. The complexes were then added drop-wise onto the cells and the cells were incubated under normal growth conditions for 24 hours. MnSOD expression with MnSOD RNAi was evaluated via Western blotting as described above, while the extent of ROS formation in MnSOD RNAi transfected cells was assessed using H₂DCF-DA and flow cytometry as described above. The effects of MnSOD-RNAi were also studied on apoptosis (Annexin V) and telomerase activity.

All data are presented as mean ± SE of separate experiments. Differences between group means were determined by a one-way ANOVA followed by a Newman Keul’s test for post-hoc comparisons. Values of P<0.05 were considered significant.
Online Supplementary Material

Figure I

The number of EPCs that underwent necrosis was greater as the concentration of CRP increased (‡P<0.05, **P<0.001). Representative images are shown below the graph. hpf=high power field. Data are represented as mean±SE and represent data from 4 independent experiments.

[Graph showing bar chart with CRP concentrations labeled as CRP 5, CRP 15, CRP 20, and Control. The y-axis is labeled as Necrotic EPC Number / HPF. The x-axis is labeled as 0, 10, 20, 30, 40. Bars are colored and shaded to indicate differences between groups.]

[Images of representative images for Control, CRP 5, CRP 15, and CRP 20 treatments.]