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C-Reactive Protein Attenuates Endothelial Progenitor Cell Survival, Differentiation, and Function

Further Evidence of a Mechanistic Link Between C-Reactive Protein and Cardiovascular Disease

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Background—Myocardial ischemia provides a potent stimulus to angiogenesis, and the mobilization and differentiation of endothelial progenitor cells (EPCs) has been shown to be important in this process. An elevated level of C-reactive protein (CRP) has emerged as one of the most powerful predictors of cardiovascular disease. However, the impact of CRP on EPC biology is unknown.

Methods and Results—EPCs were isolated from the peripheral venous blood of healthy male volunteers. Cells were cultured in endothelial cell basal medium-2 in the absence and presence of CRP (5 to 20 μg/mL), rosiglitazone (1 μmol/L), and/or vascular endothelial growth factor. EPC differentiation, survival, and function were assayed. CRP at concentrations $\geq$15 μg/mL significantly reduced EPC cell number, inhibited the expression of the endothelial cell–specific markers Tie-2, EC-lectin, and VE-cadherin, significantly increased EPC apoptosis, and impaired EPC-induced angiogenesis. EPC-induced angiogenesis was dependent on the presence of nitric oxide, and CRP treatment caused a decrease in endothelial nitric oxide synthase mRNA expression by EPCs. However, all of these detrimental CRP-mediated effects on EPCs were attenuated by pretreatment with rosiglitazone, a peroxisome proliferator–activated receptor-γ (PPARγ) agonist.

Conclusions—Human recombinant CRP, at concentrations known to predict adverse vascular outcomes, directly inhibits EPC differentiation, survival, and function, key components of angiogenesis and the response to chronic ischemia. This occurs in part via an effect of CRP to reduce EPC eNOS expression. The PPARγ agonist rosiglitazone inhibits the negative effects of CRP on EPC biology. The ability of CRP to inhibit EPC differentiation and survival may represent an important mechanism that further links inflammation to cardiovascular disease. (***Circulation***. 2004;109:2058-2067.)

Key Words: C-reactive protein ■ cells ■ apoptosis ■ nitric oxide synthase

Postnatal neovascularization is a vital compensatory response in chronic ischemia. Myocardial ischemia provides a potent stimulus to angiogenesis and the subsequent development of collateral vasculature that maintains and/or revitalizes cardiac tissue.[1–5] The mobilization and differentiation of bone marrow–derived endothelial progenitor cells (EPCs) has recently been shown to be important in this process of adult neovascularization.[6–10] Evidence suggests that EPCs contribute as much as 25% of endothelial cells (ECs) in newly formed blood vessels,[11] and transplantation of EPCs into patients has been demonstrated to induce blood flow recovery in ischemic limbs[12] and increase myocardial

viability after infarction.[13] The number and migratory activity of circulating EPCs has also been shown to inversely correlate with risk factors for coronary artery disease[14] and to serve as a surrogate biological marker for vascular function and cumulative cardiovascular risk.[15]

Accumulating evidence suggests that circulating high-sensitivity C-reactive protein (CRP) represents one of the strongest independent predictors of vascular death in a number of settings.[16–18] CRP appears to be a stronger predictor than LDL cholesterol and adds prognostic value to conventional Framingham risk assessment.[19] Initially suggested to be a biomarker, CRP now appears to be a mediator

of atherogenesis.[20] CRP has a direct effect on promoting atherosclerotic processes and EC activation.[21–27] CRP potently downregulates endothelial nitric oxide synthase (eNOS) transcription and destabilizes eNOS mRNA, which decreases both basal and stimulated nitric oxide (NO) release.[21] In a synchronous fashion, CRP has been shown to stimulate endothelin-1 and interleukin-6 release, upregulate adhesion molecules, and stimulate monocyte chemotactic protein-1 while facilitating macrophage LDL uptake.[22] More recently, CRP has been shown to facilitate EC apoptosis and inhibit angiogenesis, as well as potently upregulate nuclear factor-κB, a key nuclear factor that facilitates the transcription of numerous proatherosclerotic genes.[28] The direct proatherogenic effects of CRP extend beyond the endothelium to the vascular smooth muscle, where it directly upregulates angiotensin type 1 receptors and stimulates vascular smooth muscle migration, proliferation, neointimal formation, and reactive oxygen species production.[23] Thus, we hypothesize that the detrimental effects of CRP extend to EPCs as well.

In the present study, we tested the effects of CRP at concentrations known to predict adverse cardiac events on isolated EPC survival, differentiation, function, apoptosis, and EPC eNOS mRNA expression. We also evaluated the effects of a peroxisome proliferator–activated receptor-γ (PPARγ) agonist, rosiglitazone, on these processes in the presence and absence of CRP, because glitazones appear to have beneficial vascular effects that extend beyond augmentation of insulin sensitivity (reviewed in Wang et al[29]).

Methods

Cell Culture

EPCs were isolated by enriched medium isolation as described recently.[7] Briefly, peripheral venous blood was taken from healthy male volunteers, and the mononuclear cell fraction was isolated by Ficoll-Paque density gradient (Becton Dickinson) centrifugation and washed 3 times with PBS (Sigma), and cells were plated at a density of 10^6 mononuclear cells/cm^2 on fibronectin-coated culture slides (Becton Dickinson) in EC basal medium-2 (EBM-2; Clonetics) supplemented with 5% fetal bovine serum, with or without human vascular endothelial growth factor (VEGF)-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. Mononuclear cells were plated in the absence or presence of CRP (5 to 20 μg/mL; Trichem Resources Inc), rosiglitazone (1 μmol/L) (GlaxoSmithKline), or CRP (20 μg/mL) with rosiglitazone (1 μmol/L). The various treatments were removed after 72 hours, and EPCs were grown until day 7, with culture media changes every 48 hours.

EPC Phenotyping

EPC phenotype was determined by immunohistochemistry. EPCs were fixed in 2% paraformaldehyde (Sigma) in PBS for 10 minutes, washed 3 times with PBS, and stained with various EC-specific markers: rabbit anti-human VEGFR-2 (Alpha Diagnostics), mouse anti-human Tie-2 (Clone Ab33, Upstate Biotechnology), mouse anti-human CD34 (Becton Dickinson), EC-lectin (Ulex Europaeus Uea 1) (Sigma), and mouse anti-human factor VIII (Sigma). The presence of antibody was confirmed by exposure of the cells to an FITC-conjugated secondary antibody (either anti-mouse or anti-rabbit). Cells were mounted in VectaShield mounting medium (Vector) with propidium iodine as a nuclear marker. EPCs were then visualized under dual-emission confocal microscopy, and the percentage of cells expressing EC-specific markers was recorded. For VE-cadherin staining, cells were detached with nonenzymatic cell dissociation solution (Sigma) and were stained with FITC-conjugated rabbit anti-human VE-cadherin antibody (Serotec). Cells were analyzed with a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. The fluorescence intensity of 20 000 cells for each sample was quantified, and unstained cells were used as controls.

EPC Apoptosis Assay

The apoptotic potential of CRP on EPCs was determined by TUNEL (terminal dUTP nick end-labeling) staining. EPCs were isolated and plated on fibronectin-coated culture slides as described above for 7 days. CRP (20 μg/mL) alone or in combination with a pretreatment of 60 minutes with either rosiglitazone (PPARγ ligand, 1 μmol/L), 15d-PGJ$_2$ (PPARγ ligand, 5 μmol/L, Calbiochem), WY-14643 (PPARα ligand, 50 μmol/L, Cedarlane Laboratories), VEGF (50 ng/mL, Sigma), or Z-VAD-FMK (caspase inhibitor, 50 μmol/L, Promega) for 3 hours. EPCs were then fixed in 2% paraformaldehyde in PBS for 10 minutes and washed 3 times with PBS. EPCs were permeabilized with 0.2% Triton-X and DeadEnd Fluorometric TUNEL System staining (Promega) according to the manufacturer's recommended protocol. EPCs were then mounted in VectaShield mounting medium with propidium iodine. Cells were visualized under dual-emission confocal microscopy with all data being expressed as percent of cells undergoing apoptosis (dual stained). To exclude the possibility of apoptotic nonendothelial cells, isolated mononuclear cells were cultured as described above for 5 days, VE-cadherin–positive cells were isolated via flow cytometric cell sorting, and these cells were replated for 24 hours. The CRP group was pretreated with CRP at 20 μg/mL for 3 hours. Cell apoptosis was determined by the extent of DAPI staining.

Matrigel Tubule Assay

Growth factor reduced matrigel (Becton Dickinson) was thawed and placed in 24-well culture plates at room temperature for 30 minutes to allow solidification. DiI-acLDL (Cell Systems)–labeled EPCs (4×10^4) were coplated with 5×10^4 human saphenous vein ECs and incubated at 37°C. The number of EPCs contributing to in vitro tubule formation was assessed by counting the number of labeled cells in capillary-like structures that appeared in 5 random high-power fields per treatment group. EPCs were either pretreated with CRP (20 μg/mL), rosiglitazone (1 μmol/L), rosiglitazone (1 μmol/L) and CRP (20 μg/mL), or no additional compound (control group) for 6 days before use in the matrigel assay. To elucidate the role of NO in EPC-induced angiogenesis, EPCs were pretreated with CRP (20 μg/mL), diethylenetriamine-NO (DETA-NO; 10 μmol/L), DETA-NO (10 μmol/L) and CRP (20 μg/mL), or N^G-nitro-L-arginine methyl ester (L-NAME) for 4 days before use in the matrigel assay. Human saphenous vein ECs were cultured as described previously.[21]

eNOS and PPARγ mRNA Expression

The ability of EPCs to express eNOS and PPARγ was determined by reverse transcriptase–polymerase chain reaction (RT-PCR). EPCs were grown for 7 days in EBM-2 and exposed to CRP (20 μg/mL), rosiglitazone (1 μmol/L), or CRP (20 μg/mL) and rosiglitazone (1 μmol/L; 30-minute pretreatment with rosiglitazone) for 24 hours, after which total RNA was isolated with the GenElute mammalian total RNA kit (Sigma) and quantified by absorbance at 260 nm. Total RNA was reverse transcribed in 20-μL volumes with the Omniscript RT kit (Qiagen) with 1 μg of random primers. For each RT product, aliquots (2 to 10 μL) of the final reaction volume were amplified in 2 parallel PCR reactions with eNOS-specific (299-bp product, sense 5′-TTCCGGGGATTCTGGCAGGAG-3′, antisense 5′-GCCAT-GGTAACATCGCCGCAG-3′) or GAPDH-specific (343-bp product, sense 5′-CTCTAAGGCTGTGGGCAAGGTCAT-3′, antisense 5′-GAGATCCACCACCCTGTTGCTGTA-3′) primers and Taq polymerase (Pharmacia Biotech Amersham). PCR cycles were as follows: 94°C for 5 minutes, 65°C for 45 seconds, and 72°C for 30 seconds (35 cycles for eNOS and 25 cycles for GAPDH). RT-PCR products were analyzed by 2% agarose gel electrophoresis, visual-
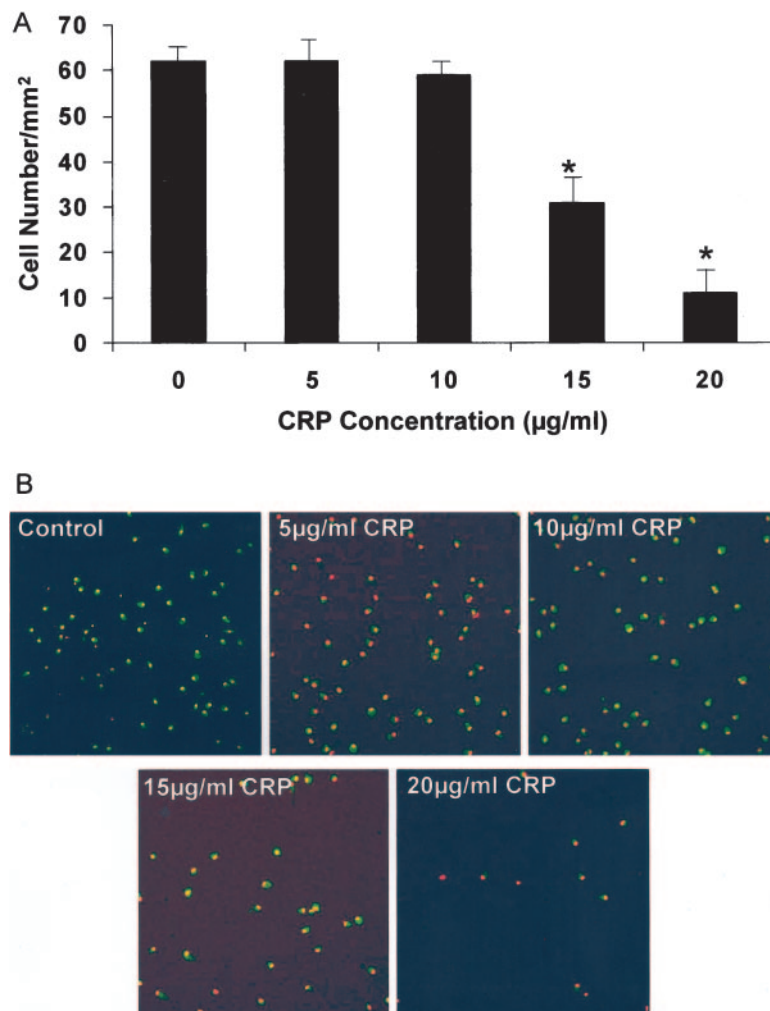
Figure 1. A, Concentration-response curve of CRP on EPC survival. EPCs were plated with increasing concentrations of CRP for 72 hours. At 15 and 20 μg/mL CRP, there was significant reduction in cell number compared with control. B, EPCs were stained with mouse anti-human VEGFR-2 antibody followed by FITC-conjugated goat anti-mouse secondary antibody. Staining was completed with propidium iodine as nuclear marker. Yellow cells are VEGFR-2 positive. *$P<0.05$ vs control.

ized with ethidium bromide, and quantified by densitometry. Results are presented as ratios between eNOS and GAPDH amplification analysis. For PPARγ mRNA analysis, total cellular RNA was isolated with the RNeasy mini kit (Qiagen), and RT-PCR was performed with the Qiagen OneStep RT-PCR kit per the manufacturer's instructions. Two micrograms of total RNA served as template for each reaction. For amplification, a primer pair specific for human PPARγ (sense primer, 5′-AGAAATGCCTTGCAGT-GGGGATGTCTCATA-3′; antisense primer, 5′-TGTAAATG-ATCTCGTGGACTCCATATTTGA-3′) was used. The primers were designed to detect all PPARγ isoforms. RT was performed at 50°C for 15 minutes. For PCR, 35 cycles were used at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The RT-PCR products were visualized on 1% agarose gels with ethidium bromide.

Statistical Analysis

All data are presented as mean±SD of separate experiments. Differences between group means were determined by a 1-way ANOVA followed by a Newman-Keuls test for post hoc comparisons. Values of $P<0.05$ were considered significant.

Results

CRP Impairs EPC Survival

To determine the effect of CRP on EPC survival, mononuclear cells were plated in the absence and presence of CRP (5 to 20 μg/mL) for 72 hours. At 7 days of growth, EPCs were characterized by positive staining for VEGFR-2 and visual-

ized by confocal microscopy. Figures 1A and 1B depict the dose-dependent effect of CRP on EPC survival. Incubation with CRP resulted in a marked dose-dependent decrease in EPC survival that was statistically significant at concentrations of CRP above 10 μg/mL (control 62±5 cells/mm²; 15 μg/mL CRP 31±8 cells/mm², $P<0.05$ versus control; 20 μg/mL CRP 11±7 cells/mm², $P<0.05$ versus control).

Rosiglitazone Treatment Improves EPC Survival

The effect of EPC treatment with the PPARγ agonist rosiglitazone was examined in the absence and presence of CRP. EPCs were cultured in EBM-2 with 5% FBS or EBM-2 with either VEGF, rosiglitazone, and/or CRP for 72 hours, followed by incubation in EBM-2. Treatment with rosiglitazone (1 μmol/L) resulted in a significant upregulation of PPARγ mRNA expression within EPCs (Figure 2A). Figures 2B and 2C depict the effect of treatment with combinations of VEGF (50 ng/mL), rosiglitazone (1 μmol/L), and CRP (20 μg/mL) on EPC survival at 7 days of growth. EPCs were characterized by positive staining for VEGFR-2 visualized by confocal microscopy. Incubation with either VEGF (163±23% increase over control, $P<0.05$) or rosiglitazone (256±48% increase over control, $P<0.01$) alone resulted in a significant increase in EPC number. Exposure to 20 μg/mL CRP resulted in a significant reduction in cell number (15±10% decrease,
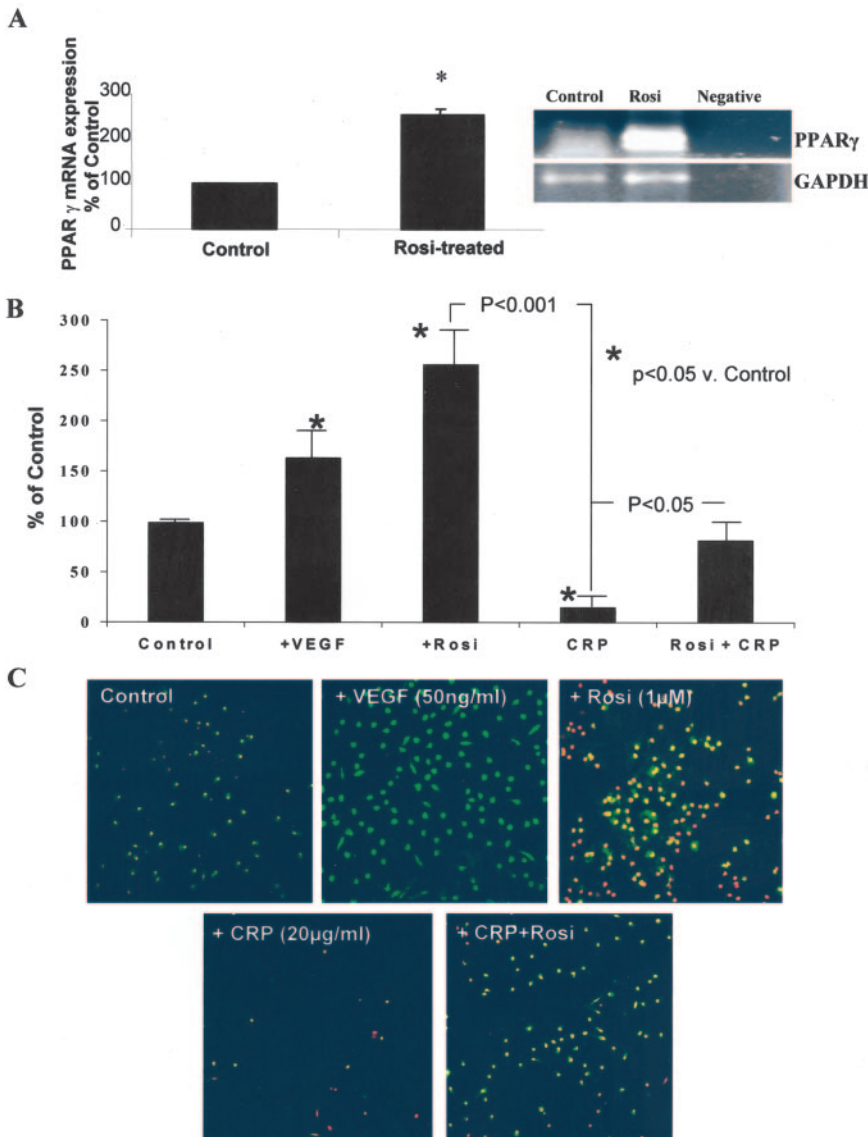
Figure 2. A, Rosiglitazone (Rosi) increases PPARγ expression in human EPCs. Human peripheral mononuclear cells were differentiated into EPCs in presence or absence of rosiglitazone (1 μmol/L) for 3 days. PPARγ mRNA expression was assessed by RT-PCR. GAPDH served as control. Results are from 3 independent experiments. *P<0.05 vs control. B, Effect of VEGF, rosiglitazone, and CRP on EPC number. EPCs were grown in either EBM-2 with 5% fetal bovine serum (Control), EBM-2 with VEGF (50 ng/mL), EBM-2 with rosiglitazone (1 μmol/L), EBM-2 with CRP (20 μg/mL), or a combination of EBM-2, CRP (20 μg/mL), and rosiglitazone (1 μmol/L). There was significant increase in cell number in both VEGF- and rosiglitazone-treated groups, and significant reduction in cell number in CRP group. Pretreatment with rosiglitazone for 30 minutes with subsequent addition of CRP resulted in significant rescue of cell number. C, All treatments were performed for 72 hours and subsequently grown up to 7 days in EBM-2. EPCs were stained with mouse anti-human VEGFR-2 antibody followed by FITC-conjugated goat anti-mouse secondary antibody. Staining was completed with propidium iodine as nuclear marker. Yellow cells are VEGFR-2 positive.

$P<0.05$ versus control, $P<0.001$ versus rosiglitazone treatment). Thirty-minute pretreatment with rosiglitazone attenuated the marked decrease in EPC survival associated with CRP ($81\pm13\%$ versus $15\pm10\%$, $P<0.05$), which resulted in a significant rescue of cell number.

CRP Increases Apoptosis of EPCs

To determine whether apoptosis is a significant mechanism underlying the effect of CRP on EPC survival, the apoptotic potential of CRP on EPCs was determined by TUNEL staining. EPCs were grown for 72 hours in EBM-2 with 5% fetal bovine serum. Cells were then exposed to CRP (20 μg/mL) with or without rosiglitazone (1 μmol/L), 15d-PGJ₂, WY-14643, VEGF (50 ng/mL), or Z-VAD-FMK for 3 hours. Figure 3A shows the percentage of cells undergoing apoptosis. There was a significant increase in CRP-mediated apoptosis of EPCs ($44\pm12\%$, $P<0.05$ versus control), as measured by TUNEL staining, which was almost completely inhibited by a 60-minute pretreatment with the PPARγ ligands rosiglitazone and 15d-PGJ₂. Treatment with the

VEGF and the caspase inhibitor zVAD-FMK, also resulted in a reversal of CRP-mediated EPC apoptosis. However, treatment with the PPARα ligand, WY-14643, did not reverse the effect of CRP, which suggests a specific effect of PPARγ. This significant proapoptotic effect of CRP was also demonstrated on a more differentiated VE-cadherin–positive cell population that excluded any nonendothelial apoptotic cells (Figure 3B).

CRP Attenuates the Expression of EC-Specific Markers

To investigate whether CRP has an impact on EPC differentiation, the effect of CRP on the expression of the EC-specific markers Tie-2, lectin, and VE-cadherin was determined. The effect of rosiglitazone pretreatment in the absence and presence of CRP was also examined. Figure 4A depicts the expression of Tie-2, and Figure 4B depicts the expression of EC-specific lectin at 7 days of growth after 72-hour treatment with VEGF (50 ng/mL), rosiglitazone (1 μmol/L), CRP (20 μg/mL), or a combination of rosiglitazone and CRP. Incuba-
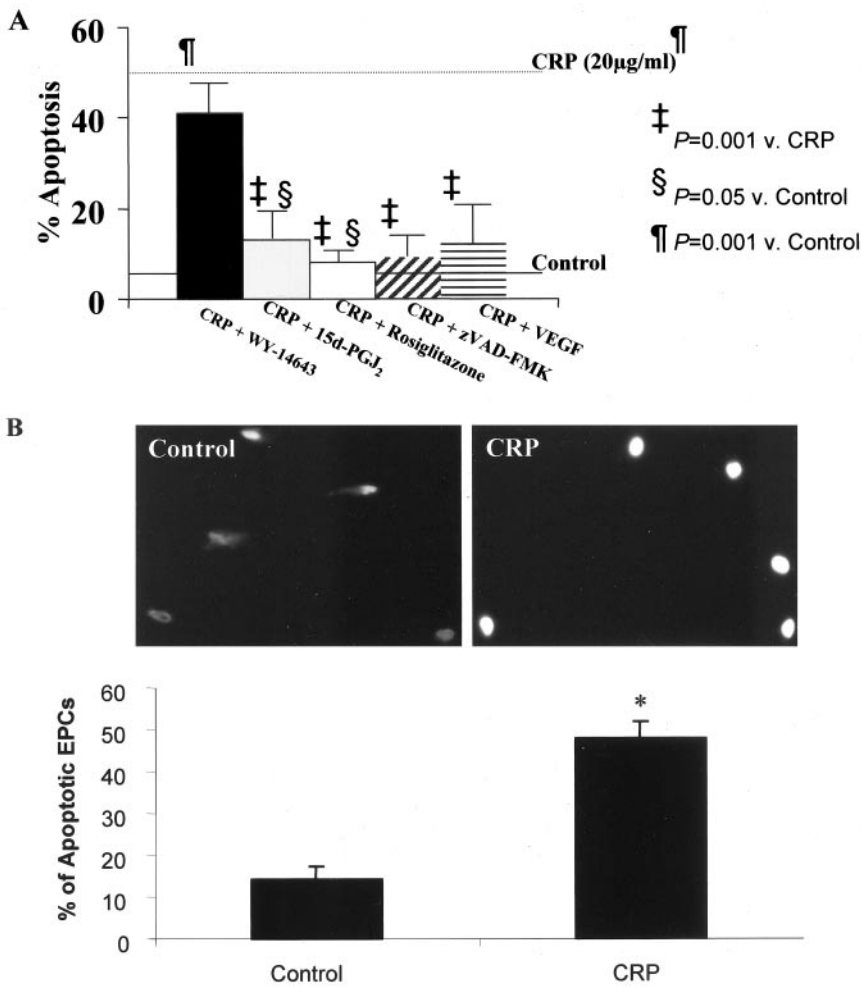
Figure 3. A, Effects of CRP with or without pretreatment of rosiglitazone (1 μmol/L), 15d-PGJ$_2$ (5 μmol/L), WY-14643 (50 μmol/L), VEGF (50 ng/mL), and Z-VAD-FMK (50 μmol/L) on EPC apoptosis. There was a significant increase in CRP (20 μg/mL)-mediated apoptosis (dotted line) of EPCs as measured by TUNEL staining that was almost completely inhibited by 60-minute pretreatment with PPARγ ligands rosiglitazone and 15d-PGJ$_2$. There was no significant inhibition of CRP-induced apoptosis by pretreatment with PPARα ligand WY-14643. General caspase inhibitor Z-VAD-FMK and VEGF both also blocked CRP-induced apoptosis of EPCs. B, Effect of CRP on VE-cadherin–positive cells. Cells were sorted by flow cytometry. Isolated cells were replated for 24 hours, and CRP group was pretreated with CRP at 20 μg/mL for 3 hours. Cell apoptosis was determined by DAPI staining. Shown is 1 representative staining. *$P<0.001$, n=4 per group.

tion with CRP attenuated the expression of both Tie-2 ($69\pm7\%$ positive versus $99\pm2\%$ positive for control) and EC-specific lectin ($64\pm6\%$ positive versus $99\pm1\%$ positive for control; $P<0.05$ versus all other groups). Pretreatment with rosiglitazone completely reversed the effect of incubation with CRP on Tie-2 ($91\pm5\%$ positive) and EC-specific lectin ($97\pm2\%$ positive) expression. CRP also decreased the number of VE-cadherin–positive cells in culture as determined by flow cytometry (Figure 4C). In the control culture, $\approx71\%$ of the cells expressed the EC-specific marker VE-cadherin. Treatment with CRP significantly reduced this number. Thus, by quantifying the expression of 3 different EC specific markers, CRP attenuated EPC differentiation. This effect was abrogated by pretreatment with rosiglitazone.

CRP Impairs EPC-Mediated Angiogenesis In Vitro

Using the matrigel tubule formation assay, we assessed the influence of CRP on EPC angiogenic function. Pretreatment with CRP (20 μg/mL) impaired the ability of EPCs to form microtubules, with fewer EPCs contributing to in vitro capillary formation. Pretreatment with rosiglitazone (1 μmol/L) significantly restored CRP impairment of EPC contribution to tubule formation; however, it was less than that observed in either the control or rosiglitazone groups (CRP 2.6 ± 2.4 versus control 46 ± 9.1, $P<0.001$; CRP

2.6 ± 2.4 versus CRP plus rosiglitazone 14.6 ± 1.2, $P<0.05$; Figures 5A and 5B). The involvement of EPCs in angiogenesis was assessed by counting the number of labeled EPCs within capillary-like tubules within 5 random high-powered fields.

EPC-Induced Angiogenesis Depends on NO Production

Treatment of EPCs with DETA-NO (10 μmol/L) attenuated the detrimental effect of CRP on in vitro angiogenesis (Figure 6). The provision of exogenous NO promoted microtubule formation within the matrigel assay, attenuating the negative effect of CRP (CRP 5.67 ± 0.88/high-powered field versus CRP plus DETA-NO 28 ± 5.69/high-powered field, $P<0.001$). However, when NO production was inhibited via the addition of L-NAME, a known inhibitor of eNOS, microtubule formation was significantly impaired (L-NAME 23.67 ± 2.41/high-powered field versus control: 58 ± 2.69/high-powered field, $P<0.001$). These results support the hypothesis that EPC-induced angiogenesis depends on NO production and that the detrimental effect of CRP may be mediated in part by interfering with its generation.

CRP Directly Attenuates EPC Expression of eNOS mRNA

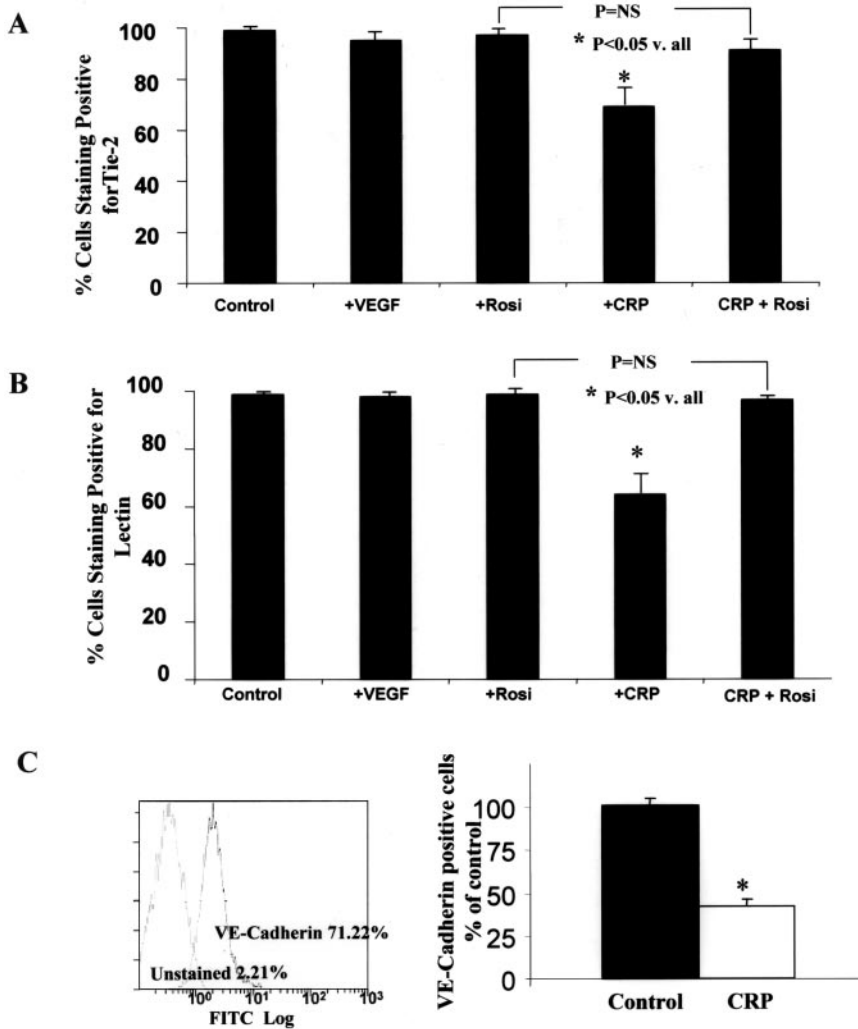The expression of eNOS mRNA was measured by semiquantitative RT-PCR. EPCs were grown for 3 or 7 days as

Figure 4. A and B, Expression of EC-specific markers Tie-2 and lectin after treatment with VEGF, rosiglitazone (Rosi), and CRP. EPCs were grown in either EBM-2 supplemented with 5% fetal bovine serum (Control), EBM-2 with VEGF (50 ng/mL), EBM-2 with rosiglitazone (1 μmol/L), EBM-2 with CRP (20 μg/mL), or combination of EBM-2, CRP (20 μg/mL), and rosiglitazone (1 μmol/L) for 72 hours. All groups were subsequently grown in EBM-2 up to 7 days. EPCs were then stained with rabbit anti-human Tie-2 antibody followed by FITC-conjugated horse anti-rabbit secondary antibody (Figure 4A) or with EC-specific lectin (Figure 4B). Staining was completed with propidium iodine as nuclear marker. Tie-2– and lectin-positive cells were counted and expressed as percentage of total cell population. C, CRP impaired differentiation of EPCs as measured by VE-cadherin expression. By flow cytometry, 71.22% of EPCs expressed VE-cadherin after culture in EBM-2. However, when treated with CRP, number of VE-cadherin–positive cells in culture was decreased significantly. *$P<0.01$.

described above and were then incubated for 24 hours with CRP (20 μg/mL) with or without a 30-minute pretreatment with rosiglitazone (1 μmol/L). Total cell RNA was isolated, amplified, and then subjected to RT-PCR with eNOS-specific primers and GAPDH-specific primers in separate reactions. Figure 7 depicts the significant decrease in EPC eNOS mRNA expression ($>$60% drop in eNOS expression, 7-day culture) as a result of incubation with CRP. Pretreatment with rosiglitazone was able to partially restore eNOS mRNA expression by EPCs. After 3 days of culture, CRP completely inhibited EPC eNOS mRNA expression.

Discussion

Given the importance of EPCs to postnatal neovascularization, we hypothesized that CRP, a powerful cardiovascular risk factor, would exert direct effects to inhibit EPC survival and differentiation. In the present study, we demonstrated that CRP has several deleterious effects on EPCs. First, EPCs incubated with human recombinant CRP, at concentrations known to predict adverse vascular outcomes, exhibited decreased survival. This reduction in EPC cell number was dose dependent, with an $\approx$80% reduction in cell number after 7 days in culture. Second, CRP promoted EPC apoptosis, which was offset by treat-

ment with PPARγ ligands. Third, EPCs incubated with human recombinant CRP exhibited decreased expression of the EC-specific markers Tie-2, EC-specific lectin, and VE-cadherin, which indicates that CRP impairs EPC differentiation. Fourth, CRP impaired in vitro angiogenesis, a measure of EPC function, a process that depends on NO. Fifth, CRP caused a significant decrease in EPC eNOS mRNA expression after 24 hours of incubation. Finally, the PPARγ agonist rosiglitazone attenuated all of the detrimental effects of CRP on EPCs. These data suggest a direct effect of CRP to inhibit EPC number and function by interfering with NO production and add to the growing body of evidence that implicates CRP as an active mediator of cardiovascular disease. In addition, we uncovered a novel role of rosiglitazone as an agent that has favorable effects on EPC biology.

Despite accumulating evidence that cardiovascular diseases such as atherosclerosis, restenosis after vascular interventions, and myocardial regeneration after infarction are influenced by bone marrow–derived cells,[6–13] the factors that influence circulating EPC number and function are just beginning to be identified. Findings that EPCs play a role in vasculogenesis have resulted in a paradigm shift in vascular biology, whereby angiogenesis, the formation of new blood
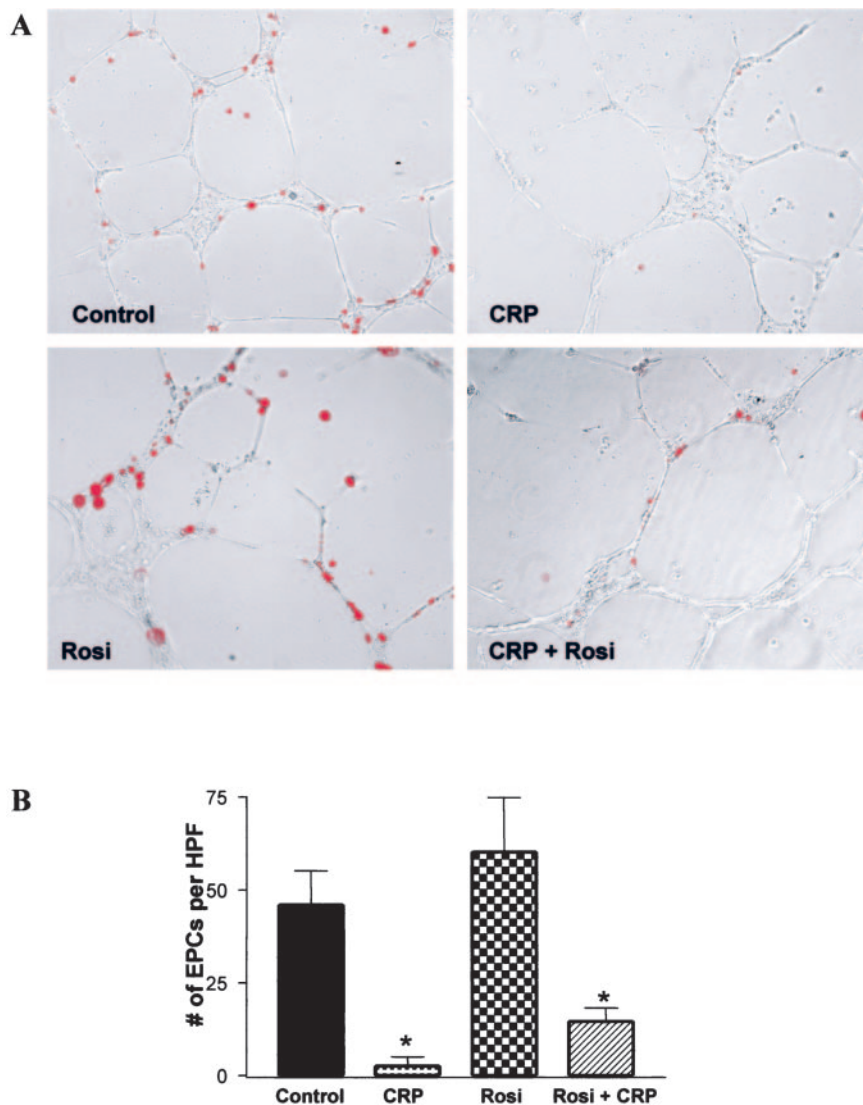
Figure 5. CRP impairs EPC-mediated angiogenesis in vitro. A, Representative images of capillary network formed in each group. Labeled EPCs appear red. EPCs were either pretreated with CRP (20 μg/mL), rosiglitazone (Rosi; 1 μmol/L), rosiglitazone (1 μmol/L) and CRP (20 μg/mL), or no additional compound (control group) for 6 days before use in matrigel assay. B, Exposure to CRP (20 μg/mL) impaired ability of EPCs to participate in tubule formation, as determined by number of labeled cells within tubules per high-powered field (HPF; CRP 2.6$\pm$2.4 vs Control 46$\pm$9.1, $P<0.001$), an effect that was partially attenuated by rosiglitazone (1 μmol/L; CRP 2.6$\pm$2.4 vs Rosi plus CRP 14.6$\pm$1.2, $P<0.05$). *$P<0.001$ vs control.

vessels from local EC proliferation, migration, and remodeling, has been redefined to include the contribution of bone marrow–derived EPCs[8] (Figure 8). The number and migratory activity of circulating EPCs is decreased in patients with risk factors for coronary artery disease, which suggests that a lack of EPCs may contribute to impaired vascularization within these patients.[14] A recent study suggested that in healthy men, levels of circulating EPCs may serve as a surrogate biological marker for vascular function and cumulative cardiovascular risk,[15] which suggests that in terms of cardiovascular diseases, an increase of EPCs could be considered a potential benefit. Also, EPC number and function are impaired in patients with type II diabetes.[30] Diabetes is well known to result in impaired coronary collateral vessel development,[31] so the discovery of EPC dysfunction in this group may contribute to the impaired neovascularization that is observed. Given the results of this study, CRP may be added to the list of factors that negatively affect EPC number.

CRP has emerged as one of the strongest independent predictors of vascular death in a number of settings.[16–18] Initially suggested to be a biomarker, CRP now appears to be a mediator of atherogenesis.[20] CRP has a direct effect on promoting atherosclerotic processes via both endothelial and smooth muscle cell activation.[21–23] The results from the present study demonstrate that CRP also exerts a detrimental effect on EPC function, the consequence of which may include impaired vessel repair and neovascularization of ischemic tissues. The cellular and molecular events involved in the regulation of EPC numbers are not yet clear. However, observations that statins increase EPC number[32,33] via a phosphatidylinositol-3 (PI3) kinase–dependent pathway that is related to the release of NO, that mice deficient in eNOS display impaired ischemia-induced angiogenesis[34] and reduced EPC mobilization,[35] and that estrogens that are known to induce NO and PI3 kinase in the endothelium[36] also increase EPC number[37] suggest that the downregulation of the NO pathway in EPCs mediated by CRP may be responsible for the detrimental effects. Our group has previously shown that CRP potently downregulates eNOS transcription and destabilizes eNOS mRNA, which decreases both basal and stimulated NO release[21] in ECs. In the present study, we demonstrate that NO is required for EPC-induced angiogen-
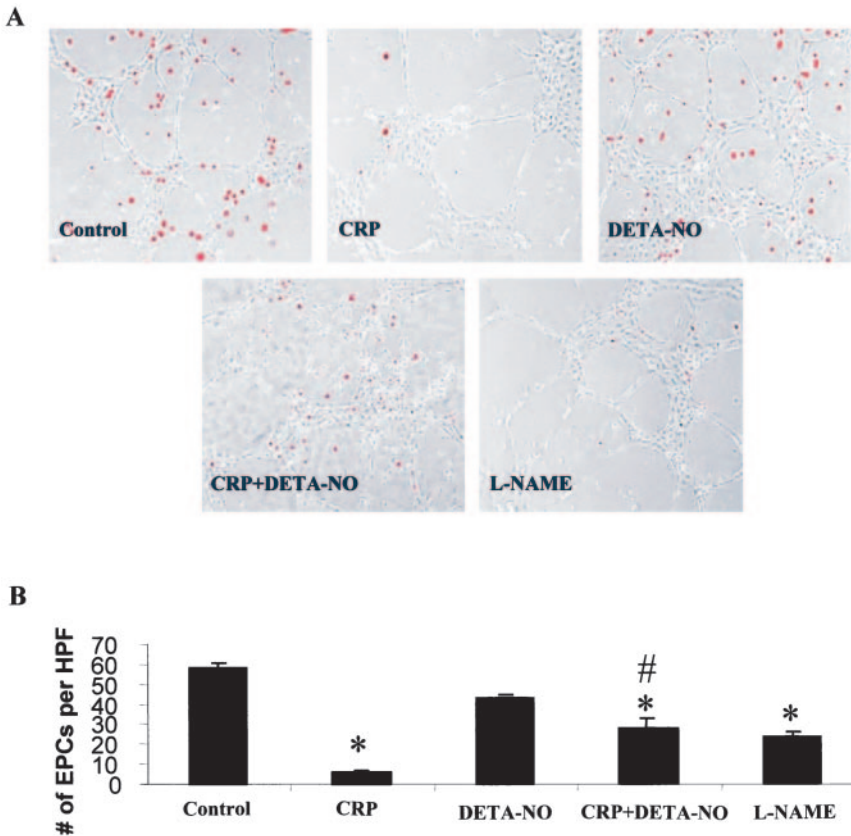
Figure 6. DETA-NO attenuated detrimental effect of CRP on in vitro EPC-induced angiogenesis. A, Representative images of capillary network formed in each group. Labeled EPCs appear red. EPCs were pretreated with CRP (20 μg/mL), DETA-NO (10 μmol/L), DETA-NO (10 μmol/L) and CRP (20 μg/mL), or L-NAME for 4 days before use in matrigel assay. B, Exposure to CRP (20 μg/mL) impaired ability of EPCs to participate in tubule formation, as determined by number of labeled cells incorporated within tubules per high-powered field (HPF; CRP 5.67$\pm$0.88 versus Control 58$\pm$2.69, $P<0.001$), an effect that was partially attenuated by DETA-NO (10 μmol/L; CRP 5.67$\pm$0.88 vs CRP + DETA-NO 28$\pm$5.69, $P<0.001$). eNOS inhibitor L-NAME significantly reduced EPC angiogenesis (L-NAME 23.67$\pm$2.41 vs Control 58$\pm$2.69, $P<0.001$). *Different from control; #different from CRP group; n=3 or 4 per group.

esis. In the presence of either CRP or the eNOS inhibitor L-NAME, microtubule formation by EPCs was reduced, a reduction that was abrogated with DETA-NO coculture. We further show that CRP decreases eNOS mRNA levels in EPCs, likely leading to a decrease in NO levels, which may promote EPC apoptosis and overall poor function.

Finally, in the present study, we demonstrated novel effects of the PPARγ agonist rosiglitazone on EPC survival, differentiation, apoptosis, angiogenesis, and eNOS mRNA production. PPARγ agonists are used to specifically augment insulin sensitivity and counter insulin resistance in diabetic patients. On ligand binding, PPARs become transcriptionally active at PPAR response elements and alter the expression of target genes. Among these targets are enhanced NO secretion by ECs[38] and suppression of nuclear factor-κB activity,[39] outcomes that favorably counter the detrimental actions of CRP. PPARγ agonists such as rosiglitazone exert beneficial effects on the cardiovascular system that extend beyond augmenta-
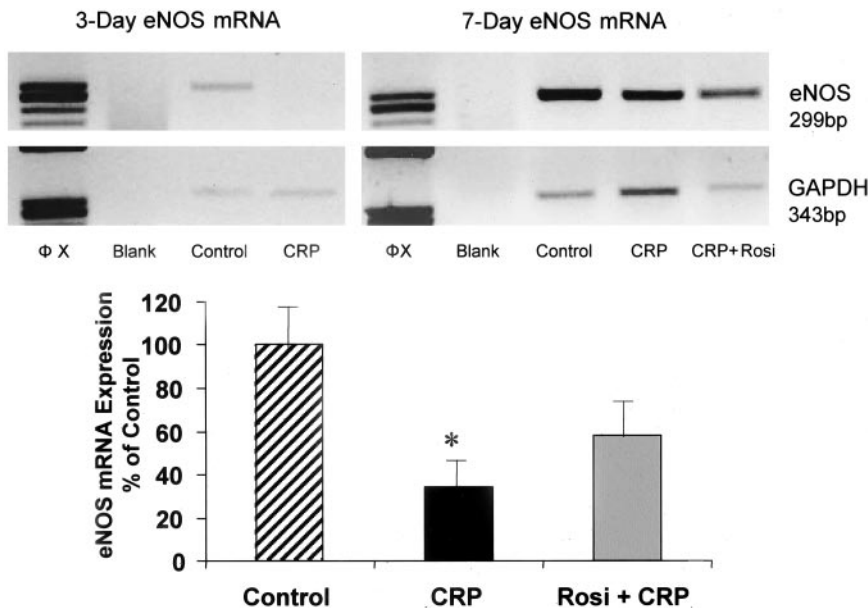

Figure 7. Effects of CRP and rosiglitazone (Rosi) on EPC expression of eNOS mRNA as measured by semiquantitative RT-PCR. EPCs were exposed to CRP (20 μg/mL) or CRP (20 μg/mL) and rosiglitazone (1 μmol/L). CRP caused 60% decrease in EPC eNOS mRNA expression (7-day culture), which was partially restored by pretreatment with rosiglitazone. After 3-day culture, CRP completely inhibited EPC eNOS expression. Degree of EPC eNOS mRNA expression represents ratio between eNOS and GAPDH mRNA. *$P<0.05$ vs Control.
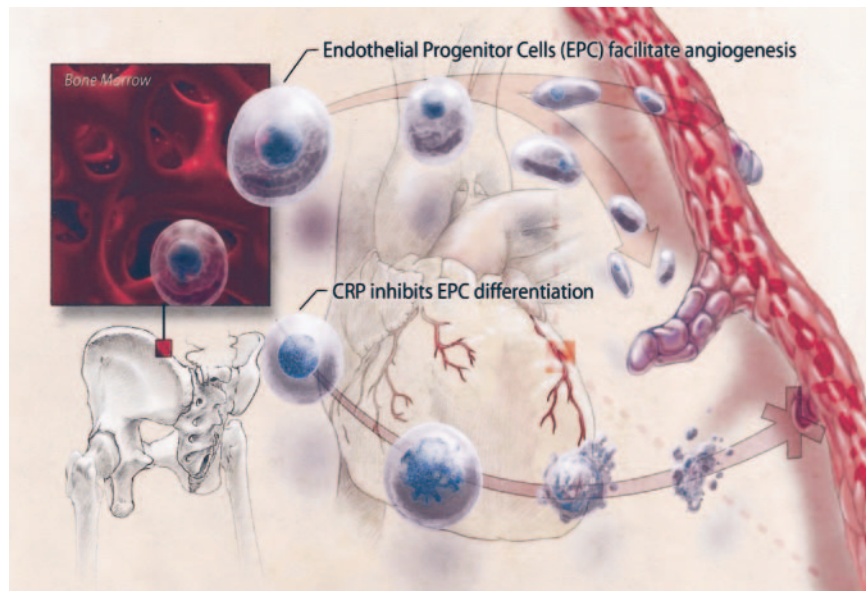
Figure 8. Bone marrow–derived EPCs contribute to postnatal neovascularization. CRP, at concentrations known to predict cardiovascular disease, inhibits EPC survival, differentiation, and function, thereby inhibiting an important mechanism of angiogenesis and compensatory response in chronic ischemia.

tion of insulin sensitivity. Glitazones appear to have a generalized antiatherosclerotic effect by attenuating endothelial dysfunction by increasing NO production,[38] inhibiting thrombin-induced endothelin-1 synthesis,[40] and interfering with leukocyte-EC interactions.[41] In addition to these vasculoprotective effects, PPARγ activators exert a positive effect on the myocardium by limiting left ventricular remodeling and failure after a myocardial infarction.[42] The results of the present study suggest that part of the beneficial cardiovascular effects observed after treatment with glitazones may be due to the favorable effect these PPARγ agonists have on EPCs. A limitation to the present study is that the in vivo effects of CRP and rosiglitazone on EPC number and vasculogenic function were not determined; however, such studies are currently being conducted. Previous studies have demonstrated the ability of rosiglitazone to lower circulating levels of high-sensitivity CRP,[43] in addition to attenuating the direct effects of CRP on EC activation.[25] The present data add to the growing body of evidence that suggests the pleiotropic vasculoprotective effects of glitazones by demonstrating their ability to increase EPC survival, differentiation, and function while counteracting the deleterious effects of CRP on these pathways.

In summary, human recombinant CRP, at concentrations known to predict adverse vascular outcomes, directly inhibits EPC differentiation, survival, and angiogenic function. This occurs in part via an effect of CRP to attenuate EPC eNOS expression. The PPARγ agonist rosiglitazone inhibits the effects of CRP on EPC differentiation and promotes EPC survival and function. The ability of CRP to negatively influence EPC biology may represent an important mechanism linking inflammation to cardiovascular disease.

Acknowledgments

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