C-Reactive Protein Enhances Tissue Factor Expression by Vascular Smooth Muscle Cells
Mechanisms and In Vivo Significance
Jianbo Wu, Meredith J. Stevenson, Jordan M. Brown, Elizabeth A. Grunz, Tammy L. Strawn, William P. Fay

Objective—We examined the impact of C-reactive protein (CRP) on vascular smooth muscle cell (VSMC) expression of tissue factor (TF) and TF pathway inhibitor (TFPI).

Methods and Results—TF mRNA, protein, and activity levels were significantly higher in VSMCs isolated from CRP-transgenic (Tg) mice than from wild-type (WT) mice. TFPI expression was significantly downregulated in CRP-Tg versus WT VSMCs. Transfection of human VSMCs with CRP expression plasmid significantly increased TF expression and decreased TFPI expression. Gene silencing of Fcγ receptor II (FcγRIIa) blocked the effect of CRP on VSMC TF expression. CRP activated p44/42, but not p38 or JNK MAP kinase (MAPK), and the effect of CRP on TF expression was blocked by pharmacological inhibitor of p44/42, but not p38 or JNK MAPK. Reactive oxygen species (ROS) scavengers blocked CRP-induced upregulation of VSMC TF expression. In vivo analyses revealed significant increases in TF expression and decreases in TFPI expression in carotid arteries of CRP-Tg mice versus WT mice.

Conclusion—CRP increases TF and decreases TFPI expression by VSMCs in vitro and in vivo. Induction of TF expression by CRP is mediated by FcγRIIIa, p44/42 MAPK, and ROS generation. These data offer important insights into the role of CRP in the pathogenesis of arterial thrombosis. (Arterioscler Thromb Vasc Biol 2008;28:698-704)

Key Words: C-reactive protein | tissue factor | VSMC | thrombosis

Elevated plasma levels of C-reactive protein (CRP) are independently associated with increased risk of myocardial infarction (MI). Several studies suggest that CRP is not simply a risk marker but also a contributing factor to vascular disease. However, CRP promotes atherosclerotic plaque formation only weakly or not at all in animal models, and plasma CRP levels correlate poorly with atherosclerotic plaque burden in humans, suggesting that CRP triggers ischemic vascular events by mechanisms other than by accelerating plaque formation. Thrombosis plays a critical role in the pathogenesis of MI and stroke. Injection of highly purified CRP into humans activates the blood coagulation system. Therefore, CRP may trigger clinical ischemic events by promoting thrombosis.

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Tissue factor (TF), a critical initiator of blood clotting, is present in atherosclerotic plaque and triggers thrombosis after plaque rupture. CRP is also present in atherosclerotic plaque, because of deposition from plasma and local synthesis by vascular smooth muscle cells (VSMCs) and macrophages. CRP stimulates TF expression by cultured VSMCs. Therefore, CRP-induced TF expression by VSMCs may promote thrombosis after endothelial disruption. However, the literature regarding the effects of CRP on vascular cells is controversial. Although several studies demonstrated that CRP induced pathological changes in vascular cells, others suggested that contaminants present in CRP preparations, such as sodium azide and lipopolysaccharide (LPS), rather than CRP itself, mediated cell activation. Furthermore, studies examining the impact of CRP on TF expression by VSMCs and other cell types have been conducted exclusively in vitro. In vivo experiments examining the effects of endogenously generated CRP, as well as in vitro experiments devoid of potential contaminating substances, are necessary to adequately examine the role of CRP in stimulating TF expression by VSMCs. Mice carrying a human CRP transgene exhibit accelerated thrombosis and increased intimal hyperplasia after vascular injury. The goals of this study were to examine the impact of CRP on TF expression by VSMCs in vitro and in vivo in CRP-transgenic (Tg) mice, and to identify mechanisms by which CRP stimulates VSMC TF expression. We also examined the effect of CRP on tissue factor pathway inhibitor (TFPI) expression by VSMCs.
Materials and Methods

Mice
C57BL/6j mice were from Jackson Labs. C57BL/6-congenic CRP-transgenic (CRP-Tg) mice were a gift from Alexander J. Szalai, PhD, University of Alabama at Birmingham.21

Reagents
SP600125 (specific inhibitor of Jun N-terminal kinase [JNK]), was from EMD Biosciences. U0126 (specific inhibitor of MAPK/extra-cellular signal regulated kinase (ERK) kinase [MEK1/2]) and SB203580 (specific inhibitor of p38 MAPK) were from Calbiochem. Purified human recombinant CRP (Trichem Resources) was dialyzed extensively against 0.1 mmol/L Tris-HCl, pH 7.5, containing 0.2 mmol/L NaCl, 2 mmol/L CaCl2, and passed over Detoxigel (Pierce). Endotoxin concentration in CRP preparations was <0.06 endotoxin units/mL. N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) were from Sigma.

Cell Culture
VSMCs were isolated from aortas of male mice22 and cultured in DMEM containing 10% fetal calf serum. Cells passed 5 to 9 times were used. Human coronary artery VSMCs were cultured in serum-containing medium provided by the supplier (Cascade). Cells passed 3 to 6 times were used.

Cell Transfection
Full-length human CRP cDNA (GeneCopoeia) was restricted with NspV and XhoI, and ligated into pReceiver-M03 plasmid (GeneCopoeia), immediately 5' to green fluorescent protein (GFP) cDNA, allowing expression of CRP as a fusion protein with C-terminal GFP (CRP-GFP plasmid). Human VSMCs were transfected with CRP-GFP or GFP (ie, pReceiver-M03, negative control) plasmid using Effectene transfection reagent (Qiagen).

Analysis of Gene Transcription
Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and digested with DNAse I (Ambion). Reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed using SuperScript 1-step RT-PCR (Invitrogen). Oligonucleotide primers were: (1) human CRP (AGCCCTCTCCATGCTTTTGG, TGTCTCTTG-GTGGCATAAGCA); (2) murine TF (GAAGAGATGGCATCT-GGCC, TACCACCTTCTTGGCTACTA); (3) murine TFPI (AA-TAACGCGAAATGGCAACC, TCGGGAAACAGCTAG-CACAGA); (4) human Fc receptor (FcCyR) Ia (ATGTTGCTTGTAACAACCTGTGC, ATGTCGTGTCTTAGCTCAAGGCCAGGA); (5) FcCyRlα (GACTCCTATTGAGCCTGTTCC, GTCACTGGTTCTAGTTGAT); (6) FcCyRlβ (GACTCCTATTGAGCCTGTTCC, CCAACAATTTGCTAGCCATCAT); and (7) FcCyRlα (AA-GATCTCCAAAAAAGGCTGTG, ATGGACCTTAGCTGACCCG).23 Positive control reactions with human leukocyte RNA (Clontech) demonstrated function of all FcCyR primer pairs. 18S RNA was amplified using universal 18S primer pair (Ambion). Band intensity of RT-PCR products was quantified with Fluochrom 8000 Imaging system (Aloha Innotech).

Gene Silencing Experiments
Nearly confluent VSMCs were transfected with siRNA duplexes (5 nmol/L) directed against human FcCyRlα (GAGAAGACUCUGGGGUAAUAdTdT, UAUAACCCAGAGUCUUCUAdTdT), FcCyRlβα (GAGACUGAGAACAGCAGUUAdTdT, UAUAUGGUCUUCACUCUAdTdT), or with negative control siRNA (Qiagen), using HiPerFect reagent (Qiagen), or with RNA duplexes directed against human p42 and p44 MAPK or negative control siRNA (Cell Signaling), according to manufacturer’s instructions.

Measurement of TF and TFPI Antigen and Activity
Cell Culture
To measure total cell TF activity, VSMCs were lysed with RIPA buffer (Sigma). Equal amounts of cell lysates were added to 96-well microtiter plates containing human factor VIIa (3 nmol/L), human factor X (100 nmol/L, Hematologic Technologies), 8.3 nmol/L CaCl2, and Spectrozyme FXa (0.33 nmol/L, American Diagnostica). After 45 minutes the absorbance of the reaction mixture at 405 nm (A405) was measured. To measure cell surface TF activity, washed VSMCs were overlaid with the above-described reaction mixture. After 45 minutes at 37°C A405 of the reaction mixture was measured. TF activity was determined from a standard curve constructed from recombinant human TF (American Diagnostica). Results are displayed as ratio of activities of experimental/control cell. Chromogenic substrate hydrolysis was negligible if factors VIIa or X were omitted. To measure secreted TFPI antigen and activity, conditioned media (CM) were collected, centrifuged to remove cells, and analyzed with Immubind TFPI ELISA and Actichrome TFPI activity assays (American Diagnostica). TFPI activity was measured as described for VSMCs.

Immunohistochemistry
Cell Culture
VSMCs were cultured on glass slides, washed, fixed, permeabilized, and blocked.24 Mouse VSMCs were incubated with rabbit IgG raised against human CRP (Santa Cruz Biotechnology) and sheep IgG raised against rabbit TF (which cross-reacts with mouse TF; American Diagnostica). After washing, slides were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit IgG against sheep IgG and Texas-Red-conjugated goat IgG against rabbit IgG (Vector Laboratories). Negative controls lacked primary antibodies. Slides were washed and incubated with 4',6-diamidino-2-phenylindole (DAPI). Human VSMCs were incubated with mouse IgG raised against human TF or mouse IgG raised against human TFPI (American Diagnostica), followed by incubation with Texas-Red-conjugated horse IgG raised against mouse IgG (Vector Laboratories).

Tissue
Carotid arteries were homogenized in 50 mmol/L Tris-HCl, pH 8.0. Protein concentration of centrifuged extracts was determined with BCA reagent (Pierce). TF activity was measured using Actichrome TF activity assay (American Diagnostica). TFPI activity was measured as described for VSMCs.

Other Assays
Western blotting was performed by subjecting VSMC lysates (25 μg total protein) to SDS-PAGE. After transfer and blocking, membranes were incubated with rabbit IgG raised against phosphorylated and nonphosphorylated forms of p44/p42, p38, and Jun–amino-terminal kinase (JNK) (Cell Signaling). Secondary antibody was horseradish-peroxidase–conjugated goat IgG raised against rabbit IgG (Santa Cruz Biotechnology). Blots were developed with ECL substrate (Pierce). Reactive oxygen species (ROS) production in VSMC lysates was measured as described.25
Statistical Analysis
Data are expressed as mean±SEM of at least triplicate experiments. Groups were compared using Student 2-tailed t test.

Results
CRP Upregulates TF Expression by VSMCs
To examine the impact of CRP on VSMC TF expression, we cultured VSMCs from wild-type (WT) mice and CRP-Tg mice. Immunofluorescence revealed significant increase in CRP expression in CRP-Tg VSMCs compared to WT VSMCs (Figure 1A, panel c versus g). TF mRNA levels, assessed by RT-PCR, were 2.5±0.1-fold higher in CRP-Tg VSMCs than in WT VSMCs (n=3/group, P<0.05, Figure 1B and 1C). Immunofluorescence analysis confirmed greater expression of TF in CRP-Tg VSMCs compared to WT VSMCs (Figure 1A, panel d versus h), cell surface TF activity was significantly greater for CRP-Tg cells than for WT cells (Figure 1D). We also examined the effect of CRP on human VSMC TF expression. Cultured human VSMCs were transfected with CRP-GFP or GFP. Nontransfected VSMCs served as an additional control. TF activity was significantly higher in cells expressing CRP-GFP (2.61±0.38 pmol/L per 10^5 cells) compared to cells expressing only GFP and nontransfected cells (2.03±0.19 and 1.66±0.28 pmol/L per 10^5 cells, respectively, P<0.05 versus CRP-GFP group). Immunofluorescence confirmed that transfection efficiency was indistinguishable between groups (data not shown).

Fcγ-Receptor IIIa Mediates Induction of TF Expression by CRP
We examined mechanisms of induction of TF expression by CRP. Because FcγRs have been implicated in cell activation by CRP,3,26 we studied expression of FcγRIa, IIa, IIb, and IIIa by RT-PCR. We found low-level expression of FcγRIIa and FcγRIIIa in human VSMCs under basal culture conditions, but did not detect expression of FcγRIa or FcγRIIb (Figure 2A). After demonstrating that exogenous CRP induced TF expression in VSMCs (Figure 2B), we examined the effect of FcγR gene silencing on CRP-induced TF expression. Human VSMCs, not transfected or transfected with control, FcγRIIa, or FcγRIIIa siRNA were grown 48 hours. CRP (50 µg/mL) or vehicle control was added to CM. Six hours later TF activity of cell lysates was measured. Only siRNA specific for FcγRIIIa significantly inhibited TF expression (Figure 2B). RT-PCR analysis confirmed that
FcγRIIa and FcγRIIIa siRNAs knocked-down expression of the intended gene (Figure 3C), without affecting expression of other FcγRs (data not shown).

**p44/42 MAPK Pathway Mediates Induction of VSMC TF Expression by CRP**

CRP induces MAPK pathway activation in VSMCs. To determine the role of MAPK activation in the induction of TF expression by CRP, we transfected human VSMCs with CRP-GFP or GFP and cultured cells, as well as nontransfected VSMCs, for 18 hours in media containing or lacking U0126 (10 μmol/L), SB203580 (0.1 μmol/L), or SP600125 (5 μmol/L). Cells lysates were prepared and subjected to Western blotting, using antibodies against p44/42, p38, and JNK MAPK. Compared to nontransfected cells, transfection of VSMCs with CRP-GFP significantly increased p44/42 phosphorylation, whereas p44/42 phosphorylation was unaffected in VSMCs transfected with GFP (Figure 3A). In VSMCs transfected with CRP-GFP, U0126 completely blocked p44/42 phosphorylation (Figure 3A). Conversely, transfection of VSMCs with CRP-GFP did not induce phosphorylation of p38 or JNK (Figure 3A; control experiments, not shown, demonstrated that antibodies recognized phosphorylated forms of p38 and JNK in positive control cell extracts [Cell Signaling]). Consistent with these data, addition of CRP (50 μg/mL) to CM of human VSMCs induced phosphorylation of p44/42, with levels peaking by 5 minutes after initial exposure (Figure 3B), but not of p38 or JNK (data not shown), and p44/42 phosphorylation was increased in murine CRP-TG VSMCs as compared to WT VSMCs (Figure 3C). To determine whether p44/42 activation triggered TF expression, human VSMCs that were either nontransfected or transfected with CRP-GFP, were grown 18 hours in presence of U0126, SB203580, SP600125, or vehicle control, after which cell lysate TF activity was measured, with results normalized to those of nontransfected, vehicle-treated cells. U0126 blocked induction of TF activity by CRP, whereas SB203580 and SP600125 had no effect (Figure 4A). To determine whether p44/42 gene silencing blocked CRP-induced upregulation of TF activity, human VSMCs were transfected with p44/42 siRNAs or control siRNA. After 24 hours, cells were transfected with CRP-GFP. Control VSMCs, not transfected with siRNA or CRP-GFP, were grown in parallel. After 24 hours, cells were lysed and TF activities, normalized to activity of control VSMCs, were measured. Transfection of VSMCs with p44/42 siRNAs significantly inhibited TF activity and

![Figure 3. CRP-induced phosphorylation of p44/42 MAPK. A, Transfection of VSMCs with CRP-GFP induced phosphorylation of p44/42, but not of p38 or JNK. B, CRP (50 μg/mL) rapidly induced phosphorylation of p44/42. C, Phosphorylation of p44/42 was increased in murine CRP-TG VSMCs as compared to WT VSMCs.](image)

![Figure 4. Inhibition of p44/42 MAPK activation blocked CRP-induced upregulation of VSMC TF expression. A, U0126, but not SB203580 or SP600125, blocked CRP-induced upregulation of TF. **P<0.05, ***P<0.6 vs control. B, siRNA knock-down of p44/42 blocked CRP-induced upregulation of TF and phosphorylation of p44/42 (inset). **P<0.05, ***P<0.1 vs control.](image)
CRP Decreases Vascular Wall TFPI Expression

We observed that in arteries of CRP-Tg mice, TFPI activity was significantly lower compared to WT mice (0.059 ± 0.003 U/mg protein, P < 0.05). Treatment with PDTC (100 μmol/L) significantly increased TFPI activity by 0.18 ± 0.01 U/mg protein in WT mice (n = 6, P < 0.05). Conversely, TFPI activity was significantly lower in carotid artery extracts of CRP-Tg mice (0.003 U/mg protein, n = 6) than for WT mice (0.18 ± 0.01 U/mg protein, n = 6, P < 0.05).

Discussion

Our study demonstrates the CRP induces TF expression by VSMCs under conditions in which sodium azide and bacterial endotoxin, which can contaminate CRP preparations and induce artifacts,6–8 were not present. We also showed that the effect of CRP on VSMC TF expression occurs in vivo in response to endogenously expressed CRP, and that CRP suppresses VSMC TFPI expression, both in vitro and in vivo. The ability of CRP to enhance TF expression, while suppressing expression of its major inhibitor, would be expected to promote thrombosis triggered by endothelial denudation. In conjunction with studies suggesting that CRP does not promote atherosclerotic plaque growth,6–10 and the observation that CRP-Tg mice exhibit accelerated thrombosis,19 our results support the hypothesis that CRP promotes thrombosis by increasing net arterial wall TF activity. TF-deficient mice exhibit attenuated VSMC migration and intimal hyperplasia after vascular injury.20 Therefore, CRP-induced increases in TF expression could also promote intimal hyperplasia. Consistent with this hypothesis, CRP-Tg mice exhibit increased intimal hyperplasia after carotid artery ligation.20

Our study offers mechanistic insights into how CRP induces VSMC TF expression. Our data suggest that FcγRIIa (CD16), a cell surface IgG Fc receptor, is expressed by VSMCs, results consistent with a recently published study.29 Furthermore, our siRNA experiments suggest that FcγRIIa mediates CRP-induced upregulation of TF expression. Devaraj et al demonstrated that FcγRIa and FcγRIIa, but not FcγRIIIa, mediate activation of human aortic endothelial cells by CRP (though this group did not examine endothelial cell TF expression).3 Therefore, there may be heterogeneity in the receptors involved in CRP-induced activation of different types of vascular wall cells. We observed that FcγRIIa is expressed by human VSMCs, results consistent with another study,27 but did not find evidence that FcγRIIa mediates upregulation of TF expression by CRP. Furthermore, mice do not express FcγRIIa,30 also supporting the conclusion that FcγRIIa mediates CRP-induced upregulation of TF expression. However, FcγRIIIa does not mediate the upregulation of TF expression by CRP. Consequently, the receptors expressed by VSMCs differ from those expressed by human aortic endothelial cells by CRP.

CRP Increases TF Expression In Vivo

To determine whether CRP increased VSMC TF expression in vivo, we analyzed carotid arteries of WT mice and CRP-Tg mice. Arterial TF mRNA (normalized to 18S) levels were increased 3.1 ± 0.3-fold in CRP-Tg mice versus WT mice (P < 0.05, Figure 5A). Immunohistochemistry revealed that TF expression was increased in the media of CRP-Tg mice (n = 3) compared to WT mice (n = 3; Figure 5B). TF activity was significantly greater in carotid artery extracts of CRP-Tg mice than those of WT mice (Figure 5C).

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Induction of TF in VSMCs by CRP Involves Oxidative Stress

Transfection of VSMCs with CRP-GFP induced ROS generation (supplemental Figure IA), results consistent with another study.27 To determine whether ROS contribute to induction of TF expression by CRP, we added NAC (10 mmol/L) or PDTC (100 μmol/L) to CM of human VSMCs 30 minutes before transfecting cells with CRP-GFP. ROS scavengers significantly inhibited induction of TF activity by CRP (supplemental Figure IB), suggesting that ROS contribute to induction of TF expression by CRP. Furthermore, our siRNA experiments suggest that FcγRIIIa mediates CRP-induced upregulation of TF expression. Devaraj et al demonstrated that FcγRIa and FcγRIIa, but not FcγRIIIa, mediate activation of human aortic endothelial cells by CRP (though this group did not examine endothelial cell TF expression).3 Therefore, there may be heterogeneity in the receptors involved in CRP-induced activation of different types of vascular wall cells. We observed that FcγRIIa is expressed by human VSMCs, results consistent with another study,27 but did not find evidence that FcγRIIa mediates upregulation of TF expression by CRP. Furthermore, mice do not express FcγRIIa,30 also supporting the conclusion that FcγRIIa does not mediate the upregulation of TF expression in VSMCs by CRP, at least in mice. A previous study suggested that CRP induces activation of extracellular signal-regulated protein kinase (ERK1/2) in phospo-p44/42 expression, as assessed by Western blotting (Figure 4B).
However, the CRP used in these experiments may have contained sodium azide, and a cause-and-effect relationship between ERK1/2 activation and TF expression was not assessed. Our data suggest that CRP induces VSMC TF expression via p44/42, but not via the p38 or JNK components of the MAPK pathway. These results clarify the intracellular signaling pathways involved in CRP-induced TF expression in VSMCs. Our studies also suggest that CRP induces TF expression in VSMCs via ROS generation, though our data do not define specific mechanisms by which ROS trigger VSMC TF expression. A study involving macrophages suggested that antioxidants inhibit TF expression by impairing translation or causing protein degradation. A recent study found that succinobucol, which possesses antioxidant properties, inhibits endotoxin-mediated induction of TF in monocytic and endothelial cells by inhibiting activation of MAPK components and transcription factors AP-1 and Egr-1.

CRP inhibits TFPI release from cultured endothelial cells, but little has been known about the effect of CRP on VSMC TFPI expression, the level of which modulates the thrombotic response to vascular injury. Our demonstration that CRP downregulates VSMC TFPI expression in vivo provides another mechanism by which CRP may promote thrombosis. Although we found approximately 3-fold increase in TF mRNA and approximately 50% reduction in TFPI activity in arteries of CRP-Tg mice, the arterial TF activity of CRP-Tg mice was only 2-fold greater than that of WT mice. These observations may be explained by the fact that TF exists in VSMCs in both active and inactive forms. Therefore, the increase in TF activity induced by CRP may be less than that suggested by the increase in TF mRNA levels. It is also possible that enhanced degradation of active TF during extraction from the arterial wall of CRP-Tg mice could result in less than anticipated increase in TF activity.

In conclusion, we demonstrated that CRP induces TF expression and inhibits TFPI expression by VSMCs, that this effect occurs in vivo, and that it occurs independently of contaminating substances that have clouded interpretation of previous studies. Our data suggest that CRP induces TF expression in VSMCs via activation of FcγRIIIa and p44/42 MAPK, and by triggering ROS generation. These data identify an important effect of CRP on the vascular wall and help to explain the prothrombotic effects of CRP. They also offer important insights into the role of CRP as a risk factor for MI. Additional studies are warranted to better define the molecular pathways by which CRP modulates TF and TFPI expression in VSMCs and to examine the effect of pharmacological suppression of CRP on TF expression and thrombosis.

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Disclosures
None.

References


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Supplemental Data

Figure I. Induction of TF expression in VSMC by CRP involved ROS production. (A) Human VSMC were transfected with GFP, CRP-GFP, or not transfected, after which ROS production was assessed by chemiluminescence. (B) ROS scavengers block induction of TF activity. VSMC were treated with NAC, PDTC, or vehicle and transfected with CRP-GFP. Non-transfected VSMC were similarly treated and cultured. After 18 hrs, TF activities of VSMC lysates were measured, with results normalized to those of vehicle-treated, non-transfected VSMC. *P<0.05 vs. other groups.